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PRINCIPAL INVESTIGATOR: Cheng Liu, M.D., Ph.D.

CONTRACTING ORGANIZATION: The Scripps Research Institute
La Jolla, CA 92037

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Cheng Liu, M.D., Ph.D.

E-Mail: chengliu@scripps.edu

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14. ABSTRACT:

Legumain is a recently discovered and only known asparaginyl endopeptidase that is well conserved throughout the biologic kingdoms. We have demonstrated that legumain is highly and inappropriately expressed in 100% human breast cancer specimens as well as murine breast cancer models. We demonstrated that an inactive prototype doxorubicin derived prodrug incorporating a succinyl blocked substrate peptide removable by legumain was effectively activated and tumoricidal in human breast cancer models. We designated this prodrug legubicin. Legubicin is not cytotoxic until activated by legumain due to reduced ability to enter cells and blocked binding to DNA. These properties led to increased tumor exposure and much reduced drug accumulation in normal tissues when administered in vivo. It has markedly reduced cardiac and myelosuppressive toxicities compare to doxorubicin. In this grant application we propose to further develop this prodrug strategy as a potential treatment for breast cancer.

15. SUBJECT TERMS
Legumain, protease, asparaginyl endopeptidase, legubicin, doxorubicin, prodrug, chemotherapy, carcinoma, breast cancer, metalloproteinase, metastasis.

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Background

Using positional gene expression profiling and high density tissue arrays, we have discovered that legumain is highly expressed by a majority of solid tumors, including 100% breast carcinomas examined [1]. Immunohistochemical analysis of tumor tissues reveals that legumain expressed by tumor cells as well as endothelial cells and tumor associated macrophages (TAM), cells constitute tumor microenvironment. In tumor microenvironment, legumain is present on cell surface in the tumor microenvironment and functional due to the acidic condition existed there. Legumain is a recently identified protease, a distinct member of the C13 family of cysteine proteases [2]. It is well conserved throughout the biologic kingdoms, found first in plants, subsequently in parasites, as well as mammals as an endopeptidase. Legumain is active in acidic pH condition and quickly inactivated under neutral pH. It has a very restricted specificity requiring an asparagine at the P1 site of substrates. Its novel specificity supports that it may be implicated in limited proteolysis consistent with limited proteolytic activation of protease zymogens, as well as selected proteins and peptides. The human legumain gene encodes a preproprotein of 433 amino acids. Mouse legumain shares 83% homology with the human protein [3]. Cells expressing legumain have enhanced migratory and invasive properties. A correlation between tumor invasion and metastasis with some cysteine endopeptidases (particularly cathepsins B and L) has been observed [4]. Legumain is critical in the activation of cathepsin B, D, and H [2, 5, 6]. We and others showed legumain activates the zymogen progelatinase A, an important mediator of extracellular matrix degradation [1] [7]. The reported inhibitory effect of cystatins on tumor cells [8, 9] is consistent with the involvement of legumain and perhaps other cysteine proteases in tumor invasion and metastasis. Tumor invasion and metastasis are the major determinants of lethality, linked to 90% of human cancer deaths [10]. The high level of legumain expression by breast cancer cells and associated cells in the tumor microenvironment coupled with its unique specificity makes it an attractive candidate for prodrug therapy for breast cancer. We reported a doxorubicin derived prodrug prepared by incorporating a peptide extension of the amino group of doxorubicin resulting in an inactive compound unless hydrolyzed to leucine-doxorubicin by an asparaginyl endoprotease [1]. This compound, legubicin, resulted in complete tumor growth arrest and eradication in a model of human breast carcinoma without toxicity, such as weight loss, myelosuppression, and cardiac toxicity in contrast to doxorubicin treated mice. Doxorubicin and related compounds are the mainstay of breast cancer chemotherapy, however its application is limited by its toxicity. In contrast to doxorubicin, legubicin do not enter cells efficiently until activated by cell surface legumain in the tumor microenvironment. Pharmacokinetics analysis and tissue distribution study support tumor specific localization and activation of legubicin. As consequence, tumor accumulation and exposure to legubicin is greatly enhanced and organ exposure to chemotherapeutic agent is reduced. In organs containing cells that normally express legumain, such as kidney and liver, no injury was evident.

Legumain expressed by kidney and liver cells are present in lysosomes and not secreted. Extracellular legumain will be quickly inactivated by neutral environment in plasma and normal tissues. Legubicin demonstrated improved efficacy profile and therapeutic index vs doxorubicin by targeting neoplastic cells as well as endothelial cells and TAM in the tumor microenvironment therefore represents a promising candidate as a first line molecularly targeted chemotherapeutic agent replacing doxorubicn for the breast cancer treatment.

Body

The funding from W81XWH-05-1-0318 provided critical support for us to pursue research in translational medicine and experimental therapeutics in the area of breast cancer. We have made significant progress in the first year of this grant cycle.

1. Development of legumain activated prodrug, legubicin, as an anti-breast cancer treatment.

A. In vivo characterization of legubicins for preclinical efficacy, therapeutic protocol advancement and safety in a panel of rodent and human breast tumors in vivo. (Months 1-36)

An advanced candidate of the prodrug was tested in variety of breast cancer models and demonstrated significant effect against primary tumor growth and metastasis. The preclinical efficacy and safety was reported in a recent Cancer Research paper titled "Targeting Cell-impermeable Prodrug Activation to Tumor Microenvironment Eradicates Multiple Drug Resistant Neoplasms" will appear in Jan. 15th issue of Cancer Research. W81XWH-05-1-0318 provided critical support for the research and was gratefully acknowledged (PDF file of the paper is attached as appendix).

Most significantly, the prodrug demonstrated significant efficacy preventing metastasis in both spontaneous and experimental metastasis.

LEG-3 prodrug suppresses experimental and spontaneous metastasis.

Treatment of LEG-3 prodrug significantly reduced experimental and spontaneous metastasis in 4T1 murine mammary carcinoma. The experimental metastasis models is generated by injecting 4T1 cells (1 x 10^5) suspended in 0.1 ml serum free medium into the tail vein of six-week-old female BALB/c mice. Treatment was given in 100-µl i.p. injections of either PBS alone (control group) or LEG-3 (100µg/100µl) daily for two weeks. The primary 4T1 models are generated by s.c. injection of 5 × 105 4T1 cells in the right flank of six-week-old BALB/c mice. Two different groups of four animals were treated between days 9 and 27 after tumor induction. Treatments were given in 100-µl i.v. injections of PBS alone (control group) or LEG-3 100µg/100µl in PBS by i.p. injection every two days for two weeks. Tumor volumes of treated animals were

measured every two-day starting on day 9 by microcaliper measurements (volume = width × length × width/2). As soon as the tumor volume reached 1400 mm^3 in the control groups (on day 30th), euthanasia was performed and lungs were removed and fixed in the Bouin's solution. Lung metastases were counted by anatomy microscope. Statistical significance between treatment groups was determined by two-tailed Student's t tests using Microsoft EXCEL software.

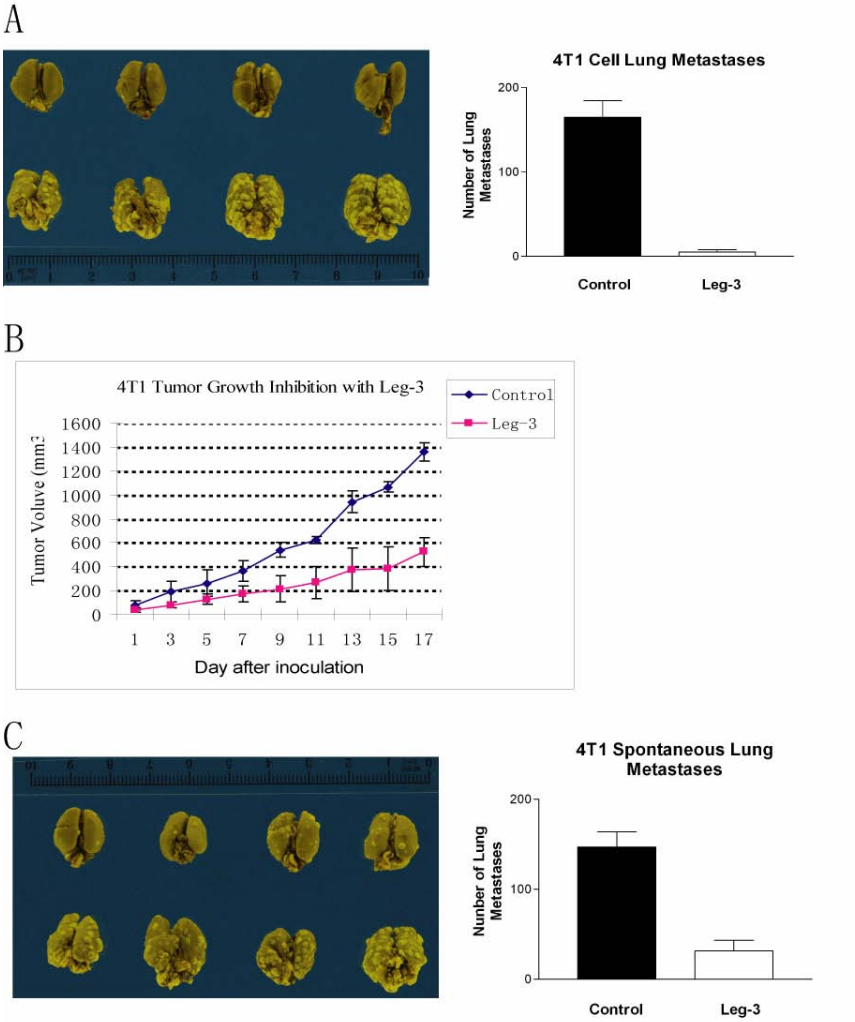


Figure 1. *LEG-3 suppresses experimental and spontaneous metastasis. (A) Experimental lung metastasis in treatment group (top) versus control group (bottom). (p<0.005). (B) Mouse mammary carcinoma growth suppression by LEG-3 (n=8). (C) LEG-3 treatment significantly reduces spontaneous lung metastasis in 4T1 tumors. (p<0.005).*

A provisional patent titled "Inhibiting Tumor Cell Invasion, Metastasis, and Angiogenesis" was filed Nov. 29, 2005. (PDF file is attached).

B. Legumain specificity explored through phage displayed substrate peptide library and synthetic peptide. (Months 1-6)

Due to the success with the current candidate compound, the construction of phage library is delayed until the second year of grant.

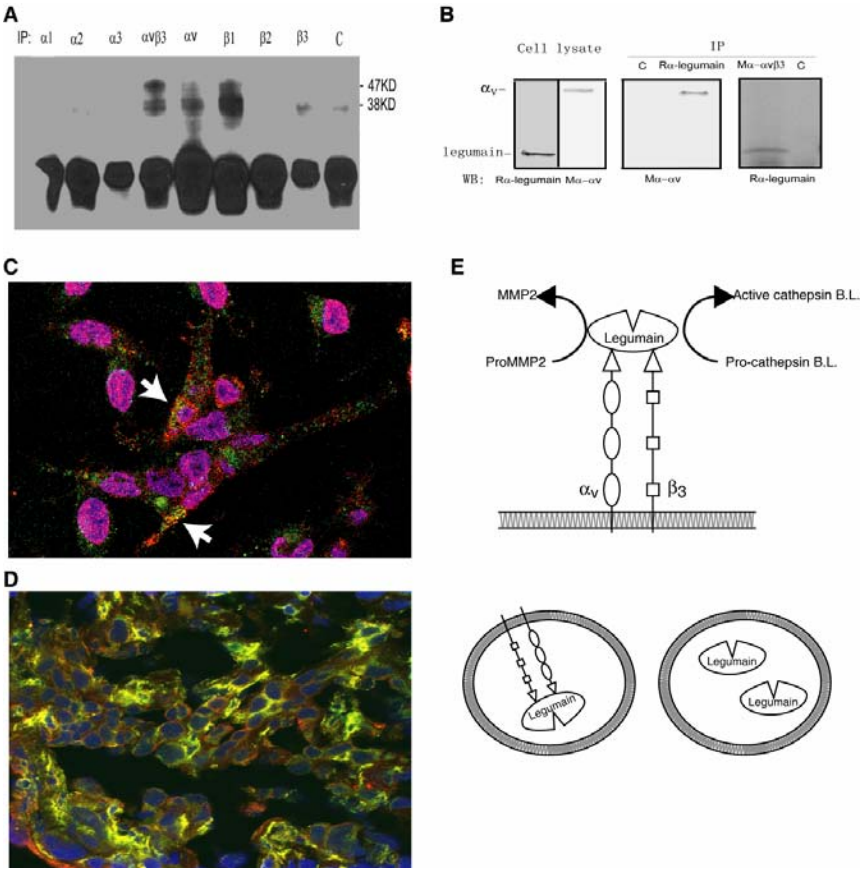
C. Improving chemistry and synthesis of legubicins, and efficient analysis of designed peptidyl and pseudopeptidyl derivative structures for improved efficacy and safety of legubicin analogues using in vitro assays. (Months 1-36)

We have synthesized different versions of the prodrug including dimmer the prodrug. And these candidate compounds will be assessed for their in vitro and in vivo efficacies as proposed in the grant.

2. <u>Characterization of the molecular cell biology of legumain in breast cancer biology</u>.

A. Producing legumain knockdown and over-expression cells and investigate the effect of legumain knockdown and over-expression on breast cancer cells migration invasion in vitro. (Months 1-12)

We have produced multiple breast cancer cell lines that are over-expressing legumain as well as expressing legumain shRNA that suppresses legumain expression. Our data support the role of legumain in cell migration and invasion. Therefore this aim is accomplished. These work led to new hypothesis of mechanisms that legumain modulate tumor microenvironment.



B. Investigate the effect of legumain knockdown and over-expression on breast cancer invasion/metastasis in animal models. (Months 6-18)

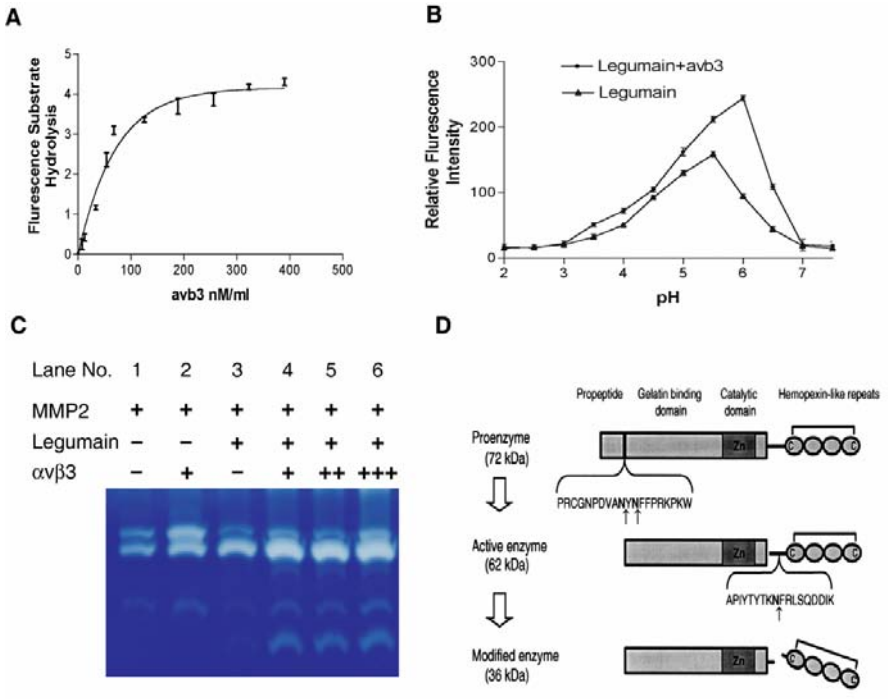
Continuing from the last aim, We discovered that legumain:αvβ3 protease complex regulate cell surface proteolysis.

Figure 2. legumain:αvβ3 form protease complex. (A) legumain is immuno-precipitated with a panel of anti-integrin antibodies. (B) Immuno-precipitation of legumain by anti αvβ3 antibody and αvβ3 by anti-legumain antibody. (C) legumain:αvβ3 complex in MBA-MA231 cells. αvβ3 is red, legumain is green, nuclei is blue. And legumain:αvβ3 complex is yellow. They are formed intracellularly in vesicles and transported to cell surfaces. (D) Extensive legumain:αvβ3 in Panc-1 human pancreatic carcinoma grow in nude mice.

The αvβ3 is red, legumain is green, nuclei are blue. The legumain:αvβ3 co-localization in complex is yellow. (E) Schematic representation of the legumain:αvβ3 complexe in intracellular trafficking and cell surface proteolysis.

Legumain is distributed intracellularly as well as presented on cell surfaces in the tumor microenvironment via binding to integrins, particularly the αvβ3 subtype. The legumain:αvβ3 complex forms intracellularly and are transported together in vesicles to the leading edge of migrating cells (Figure 2). Binding of legumain to αvβ3 integrins significantly enhanced its enzymatic activity and ability to activate its physiologic substrate such as pro-gelatinase A and pro-cathepsin B (Figure 3). We provided evidences to support that legumain may provide the necessary proteolytic activity in the legumain: $\alpha_v\beta_3$ not only activate MMP-2 and can also generate the hemopexin fragment, a known inhibitory molecule for angiogenesis. These results indicate that the legumain:αvβ3 complex is an important modulator of pericellular proteolysis during tumor invasive growth and angiogenesis. The enhanced legumain amidolytic activity indicates the cell surface legumain:αvβ3 complex is likely the primary target enzyme of tumor microenvironment activated prodrug.

Figure 3. Integrin αvβ3 is co-factor of legumain activity. (A) Binding of αvβ3 increases the amidolytic activity of legumain. (B) The αvβ3 integrin also shift the pH dependency of legumain. (C) The αvβ3 increases its activity towards its physiologic substrate MMP2 as assessed using zymogram. (D) Schematic illustration of MMP2 activation by legumain. Legumain not only activate MMP2 by removing its propeptide, it also cleave between its catalytic domain and hemopexin-like repeats, later a known inhibitory molecule for angiogenesis, support a regulatory role of legumain during vessel development.



Part of the data included in the grant and here was presented at “Cancer, Protease, and the Tumor microenvironement, Nov. 30-Dec. 4, 2005” in an abstract titled “Hypoxia-induced Legumain Expression and Localization to Invasive Cell Surface via Co-factor αvβ3 Integrins Is Critical for Tumor Invasion and Angiogenesis”.

C. Legubicin action against tumor endothelial cells and tumor associated macrophages. (Months 18-36)

These experiments are undergoing and will be achieved on time.

Key Research Accomplishments

1. Demonstrated efficacy of TME activated prodrug, legubicin, against drug resistant breast cancers.

2. Elucidation of the mechanism of legumain in promoting metastasis/invasion and angiogenesis.

Reportable Outcomes

1. Prove of principle of tumor microenvironment activated prodrugs and in vivo demonstration of prodrug efficacy against drug resistant breast cancers (paper published in Cancer Research).

2. The prodrug demonstrated significant anti-cancer efficacy and the data generated from the first year formed a base of a new provisional patent.

Conclusions

The local tumor microenvironment differs greatly from that of other tissues. One key character is that it is enriched in proteolytic activity. Cell surface proteases, such as legumain, play important role in cancer progression such as invasion/metastasis and angiogenesis. The over-expression of these cell surface proteases are ideal physical as well as functional targets to activate prodrugs in the tumor microenvironment as demonstrated by data generated with the support of this grant.

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Appendices

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Targeting Cell-Impermeable Prodrug Activation to Tumor Microenvironment Eradicates Multiple Drug-Resistant Neoplasms

Wenyuan Wu,[1] Yunping Luo,[1] Chengzao Sun,[2] Yuan Liu,[1] Paul Kuo,[1] Janos Varga,[3] Rong Xiang,[1] Ralph Reisfeld,[1] Kim D. Janda,[2] Thomas S. Edgington,[1] and Cheng Liu[1]

Departments of [1]Immunology and [2]Chemistry, The Scripps Research Institute, La Jolla, California; and [3]California Peptide Research, Inc., Napa, California

Abstract

The tumor microenvironment is notably enriched with a broad spectrum of proteases. The proteolytic specificities of peptide substrates provide modular chemical tools for the rational design of cell-impermeable prodrugs that are specifically activated by proteases extracellularly in the tumor microenvironment. Targeting cell-impermeable prodrug activation to tumor microenvironment will significantly reduce drug toxicity to normal tissues. The activated prodrug attacks both tumor and stroma cells through a "bystander effect" without selectively deleting target-producing cells, therefore further minimizing resistance and toxicity. Here, we showed that legumain, the only asparaginyl endopeptidase of the mammalian genome, is highly expressed by neoplastic, stromal, and endothelial cells in solid tumors. Legumain is present extracellularly in the tumor microenvironment, associated with matrix as well as cell surfaces and functional locally in the reduced pH of the tumor microenvironment. A novel legumain-activated, cell-impermeable doxorubicin prodrug LEG-3 was designed to be activated exclusively in the tumor microenvironment. Upon administration, there is a profound increase of the end-product doxorubicin in nuclei of cells in tumors but little in other tissues. This tumor microenvironment–activated prodrug completely arrested growth of a variety of neoplasms, including multidrug-resistant tumor *in vivo* and significantly extended survival without evidence of myelosuppression or cardiac toxicity. The tumor microenvironment–activated prodrug design can be extended to other proteases and chemotherapeutic compounds and provides new potentials for the rational development of more effective functionally targeted cancer therapeutics. (Cancer Res 2006; 66(2): 970-80)

Introduction

The local tumor microenvironment differs greatly from that of other tissues. It is enriched in proteolytic activity, is acidic, and is hypoxic. It represents a favorable environment for protease-targeted conversion of inactive compounds to potent cytotoxic agents (1). Targeted activation of prodrug in the tumor extracellular microenvironment can reduce the pressure for selection of tumor cells lacking expression of the target enzyme, and thereby enhance safety and efficacy of tumor destruction. This goal may be achieved by incorporation of cell membrane permeability inhibiting groups that are subject to catalytic removal by a targeted enzyme present and active in the tumor extracellular microenvironment. Such cell-impermeable targeting prodrugs promise to profoundly reduce active drug access to normal tissues, enhance availability to tumors, and therefore significantly reduce toxicity to normal cells and enhance destruction of neoplastic cells.

Legumain is an entirely novel evolutionary offshoot of the C13 family of cysteine proteases (2). It is well conserved from plants to mammals, including humans. First identified in plants as a processing enzyme of storage proteins during seed germination (3, 4), it was subsequently identified in parasites and then in mammals (5). Legumain is a robust acidic cysteine endopeptidase with remarkably restricted specificity absolutely requiring an asparagine at the P1 site of substrate sequence (5). We found legumain to be highly expressed in a majority of tumors, including carcinomas of the breast, colon, and prostate, and in several central nervous system neoplasms (6); on the other hand, expression is not apparent in normal tissues from which the tumors originated. Legumain is present intracellularly in endosome/lysosome systems and is associated with intracellular protein degradation. Importantly, we showed that legumain is also present extracellularly in the tumor microenvironment, associated with matrix as well as cell surfaces and functional locally in the reduced pH of the tumor microenvironment. Although evident in tumors, this endopeptidase is not detectable in the same tumor cell lines in culture that are used to generate the *in vivo* tumors, inferring an induction of legumain gene expression by the tumor microenvironment (7, 8). In addition to neoplastic cells, we found that legumain is expressed by tumor angiogenic endothelial cells and also here show presence in and on tumor-associated macrophages (9–11), thus presenting multiple local intratumoral cellular targets for prodrug activation (12).

In view of these attractive properties, we designed several legumain-activated prodrugs by covalently linking a cell-impermeable succinyl blocked substrate peptide to the aminoglycoside of doxorubicin. This prototype cell-impermeable targeting tumor microenvironment–activated prodrug is inactive and nontoxic until activated extracellularly by legumain in the acidic tumor microenvironment. Based on *in vivo* activity, LEG-3 (*N*-succinyl-β-alanyl-L-alanyl-L-asparaginyl-L-leucyl-doxorubicin) possessed profoundly reduced toxicity and markedly enhanced efficacy compared with doxorubicin in both murine syngeneic CT26 colon cancer and C1300 neuroblastoma models as well as in the human fibrosarcoma HT1080 and doxorubicin-resistant prostate cancer MDA-PCa-2b xenograft models. Mechanistic and pharmacokinetic evidence support the tumor microenvironment–activated prodrug strategy.

Note: W. Wu and Y. Luo contributed equally to this work.

Requests for reprints: Cheng Liu, Department of Immunology, The Scripps Research Institute, 10550 North Torrey Pines Road, SP258, La Jolla, CA 92037. Phone: 858-785-7734; Fax: 858-785-7756; E-mail: chengliu@scripps.edu.

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The potent *in vivo* antitumor efficacy and the improved therapeutic index suggest that LEG-3 represents a promising candidate for highly selective chemotherapeutic eradication of tumors.

Materials and Methods

Reagents and cell lines. Rabbit polyclonal antisera against human legumain as well as 293 cells stably expressing human legumain were kindly provided by Dr. D. Roodman (Department of Medicine and Hematology, University of Texas Health Science Center, San Antonio, TX). A legumain substrate peptide was purchased from Bachem, Inc. (King of Prussia, PA). Doxorubicin was purchased from Sigma (St. Louis, MO) and DMEM was from Invitrogen (Carlsbad, CA). The CT26 murine colon carcinoma, C1300 mouse neuroblastoma cell lines, and the human HT1080 fibrosarcoma cells were purchased from American Type Culture Collection (Manassas, VA).

Antibody preparation. Antilegumain antibodies were prepared by immunizing rabbits with keyhole limpet hemocyanin–conjugated peptide CGMKRASSPVPLPP. A cysteine is added to the legumain sequence. The antilegumain antibodies were affinity purified from resultant antisera using peptide antigen coupled to Ultralink Iodoacetyl Gel from Pierce (Rockford, IL). The bound antibodies were eluted by glycine buffer (100 nmol/L, pH 2.7) and neutralized immediately by adding one-tenth volume of 1 mol/L Tris (pH 7.5).

Western blot. Proteins were dissolved in 2× SDS sample buffer for SDS-PAGE analysis using gradient Tris-glycine gels (8-16%). After electrophoresis, the proteins were transferred to nitrocellulose membranes and blocked with nonfat milk. The antilegumain antiserum was used as the first antibody and was incubated with the membranes for 1 hour (1:1,000 dilution). The blot was washed thrice with PBS, incubated with streptavidin-peroxidase for 15 minutes, and developed by the enhanced chemiluminescence method (Sigma).

Flow cytometry analysis. Single-cell suspensions were prepared from organs and tumor tissues as previously reported (6). Rabbit antilegumain antisera diluted 1:5,000 or antigen purified antilegumain antibody at 0.5 μg/mL in PBS are used to detect legumain. This is followed by FITC-conjugated goat anti-rabbit IgG diluted 1:5,000 in PBS (BD PharMingen, La Jolla, CA). For CD14 staining, the phycoerythrin-conjugated anti-mouse CD14 antibody diluted 1:3,000 in PBS was used (BD PharMingen).

Immunohistochemical analysis. Immunohistochemical staining was done on 5-μm-thick frozen sections on poly-L-lysine slides. For endothelial identification, biotinylated rat anti-mouse CD31 monoclonal antibody (MEC 13.3) was used with Texas red–conjugated streptavidin as the secondary reporting reagent. For staining of legumain, rabbit polyclonal anti-legumain antisera was used at 1:500 dilution or antigen-purified antilegumain polyclonal antibody at 0.5 μg/mL and visualized with FITC-conjugated goat anti-rabbit antibody. For the identification of tumor-associated macrophage, rat anti-mouse CD68 antibody was used and followed by an antirat antibody conjugated with Texas red. For identification of collagen I, a biotinylated rabbit anti-mouse collagen I antibody was used at 1:250 dilution and visualized with Texas red–conjugated streptavidin. The slides were analyzed by laser scanning confocal microscope (Bio-Rad, Hercules, CA).

Terminal deoxynucleotidyl transferase–mediated nick end labeling analysis. DNA fragmentation caused by apoptosis was detected by terminal deoxynucleotide transferase–based, *in situ* cell death detection kit (Roche Applied Science, Mannheim, Germany). The procedure was done according to the instructions of the manufacturer. Briefly, the sections were treated with protein K solution (10 μg/mL in 10 mmol/L Tris/HCl, pH 7.4) for 15 minutes and followed by 15-minute incubation with terminal deoxynucleotidyl transferase (TdT)–mediated nick end labeling (TUNEL) reaction mixture containing TdT and FITC-dUTP. The TUNEL alkaline phosphatase kit (Roche Applied Science) was used for the conversion of fluorescence-based TUNEL detection into a colorimetric labeling. The conversion was achieved by binding of an antifluorescein antibody to FITC-dUTP. The antibody is labeled with alkaline phosphatase. The signals were visualized with Fast Red (Vector Laboratories, Burlingame, CA).

Prodrug synthesis. The synthesis of the succinyl version of the prodrug used the azide method to protect the peptide from racemization. In principle, the N-protected amino acids or peptide esters are converted by hydrazine derivatization to an acid hydrazide. Subsequent reaction with HNO_2 or derivatives leads to anacylazide. Thus, the succinyl-Ala-Ala-Asn-Leu-N_2H_3 peptide was prepared by using liquid phase synthesis. It was directly used to synthesize the target compound. An example of the synthesis is as follows. Solution A: 1,040 mg succinyl-Ala-Ala-Asn-Leu-N_2H_2F was dissolved in a small amount of dimethylformamide (DMF) cooled to −10°C and 1.5 mL of 4 N HCl dioxane was added followed by 2.1 mmol/L isoamylnitrite. The mixture was stirred for 30 to 40 minutes at −10°C and then the pH was carefully adjusted to 7.5 with diisopropyl ethylamine. Solution B: 1,210 mg doxorubicin acetate was dissolved in a small amount of DMF at room temperature, the pH was adjusted to 7.5 with DIPEA, and the solution was chilled to −10°C. Solutions A and B were combined and the pH was readjusted to 7.5 and monitored throughout the reaction. The reaction mixture was allowed to warm to 4°C and allowed to stand overnight. High-performance liquid chromatography (HPLC) analysis indicated ~80% completion of the reaction within 24 to 48 hours. The reaction mixture was then diluted 10-fold with 0.1% trifluoroacetic acid (in H_2O) and applied directly onto preparative HPLC. A linear acetonitrile gradient was used to elute the target compound. Fractions were analyzed for purity, combined, and lyophilized. HPLC, amino acid analysis, and mass spectrometry were done on the lyophilized powder.

Cytotoxicity assays. The WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio-1,3-benzene disfonate) cell proliferation reagent (Roche Molecular Chemicals, Mannheim, Germany) was used to determine cell proliferation by quantization of cellular metabolic activity. Control 293 cells and legumain$^+$ 293 cells were cultivated in microtiter plates (5×10^3 per well in 100 μL) and were incubated with serial concentrations of LEG or doxorubicin for 48 hours. Subsequently, 10 μL WST-1 solution (1 mg/mL WST-1, 25 μmol/L methyldibenzopyrazine methyl sulfate) was added per well and mixtures were incubated for an additional 4 hours. The tetrazolium salt WST-1 was cleaved by the mitochondrial succinate-tetrazolium-reductase system to formazan in cells in direct correlation with the number of metabolically viable cells in the culture. The amount of formazan salt was quantified in three replicates by absorbance at 450 nm using a microplate reader (Molecular Devices, Palo Alto, CA). All results were derived from replicate experiments with similar results.

Doxorubicin and LEG-3 uptake assay. The legumain$^+$ 293 cells or control 293 cells (2.5×10^5 per well) were seeded in six-well plates. The culture plates were then incubated for 24 hours at 37°C and 5% CO_2 and the medium in each well was replaced with 2 mL of serum-free, antibiotic-free medium containing various concentrations of doxorubicin or LEG-type compounds. The cells were incubated 1.5 hours then washed thrice with 2 mL cold PBS. At this point, cell nuclei doxorubicin positivity can be analyzed by fluorescent microscopy. For quantitative assays, the cells were then lysed by adding 0.5 of water and gently rotated on an orbital shaker for 5 minutes at room temperature. The lysed cells were added to 1.5 mL acidified ethanol and incubated at 4°C in the dark for 3 hours. Total doxorubicin and LEG content was measured fluorometrically using a Perkin-Elmer LS-50-B spectrofluorometer (excitation: 470 nm; emission: 590 nm). Fluorescence intensity was translated to drug concentration by use of a standard curve prepared from doxorubicin and LEG solutions in cell lysates that were not previously exposed to the drug. Results are expressed as the mean ± SD of at least three replicates for each experiment.

Determination of marrow toxicity. Groups of healthy BALB/c mice ($n = 4$) were injected i.p. with a single dose of LEG-3 (49.4 or 4.94 μmol/kg) or doxorubicin (3.4 μmol/kg). On day 7, retro-orbital sinus blood samples were collected into 10 mmol/L EDTA and were counted by hemocytometer after lysis of RBCs with an acidified methyl violet solution.

Determination of maximum tolerable dose. Four six-week-old BALB/c mice were used for each experimental group. The mice were weighed individually and the average weight of the group is used to determine the exact doses. Mice were given i.p. injection daily for 5 days. The maximum tolerable dose (MTD) is defined as the maximum drug dose administered to non–tumor-bearing mice once daily for 5 consecutive days without mortality.

Tissue distribution. LEG compounds or doxorubicin was injected i.p. into mice; 12 hours later, the animals were perfused and the doxorubicin autofluorescence was measured following homogenization in 50% ethanol and then diluted with an equal volume of 50% ethanol containing 0.6 mol/L HCl. Fluorescence measurements were obtained with excitation at 470 nm and emission at 590 nm; concentrations were derived by conversion from a doxorubicin standard curve. Tissues from saline-injected mice provided controls. Blood samples were to 0.75 mL with PBS, centrifuged, the pellets washed with PBS, and doxorubicin was extracted with ethanol and 0.3 mol/L HCl.

Animal models. The CT26 syngeneic murine colon carcinoma model was generated and maintained in The Scripps Research Institute animal facility. This model was produced in BALB/c mice ages 4 to 6 weeks injected with 5×10^5 CT26 tumor cells per s.c. site on the back. The C1300 mouse neuroblastoma model was generated in A/J mice by s.c. injection of 5×10^5 C1300 cells per site on the back. Treatment was initiated when the tumors reached 4 mm in diameter through bolus i.p. (syngeneic tumors) or i.v. (human tumors) injections of the indicated reagents. Treatment was thrice per week for 2 weeks. The human HT1080 fibrosarcoma was xenografted in BALB/c *nu/nu* mice obtained from The Scripps Research Institute breeding colony. HT1080 cells, 1×10^6 per site, were inoculated s.c. on the back. The MDA-PCa-2b human prostate carcinoma model was generated in WEHI nude mice and these cells (10^6) were also injected s.c. Tumor growth and signs of physical discomfort were monitored daily including for any gross evidence of tumor necrosis, local tumor ulceration, as well as evidence of toxicity including the mobility of animals, response to stimulus, piloerection, eating, and weight. These procedures have been reviewed and approved by the Institutional Animal Care and Use Committee at The Scripps Research Institute. All the experiments were conducted in The Scripps Research Institute facilities, which are accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. The Scripps Research Institute maintains an assurance with the Public Health Service and is registered with the Department of Agriculture and is in compliance with all regulations relating to animal care and welfare.

Statistical analysis. Statistical significance of data was determined by the two-tailed Student's *t* test, except for statistical significance of survival curves, which used the log-rank test using GraphPad Prism version 3.00 (GraphPad Software, San Diego, CA).

Results

Legumain is highly expressed by cells in the tumor microenvironment. We have reported that legumain is expressed *in vivo* by tumor cells and proliferating endothelial cells intracellularly as well as on their cell surfaces (6). In contrast, legumain is not detectable in or on CT26, C1300, HT1080, and MDA-PCa-2b as well as eight other tumor cell lines in culture, the same cells used to generate a set of legumain-expressing tumors *in vivo* (Fig. 1*A*). Further, this endopeptidase is not detectable in large-scale survey panels of tumor cell lines in culture, except for THP-1 cells, based on a search of the National Cancer Institute tumor cell expression database.

Using immunohistochemical analysis, legumain is usually present in tissue sections of human colon carcinomas, neuroblastomas, fibrosarcomas, and prostate carcinomas, representative of the types of neoplasms investigated in the present study. Legumain expression was not detectable in the normal tissues of origin for these neoplasms (6). The remarkable local expression of legumain not only by neoplastic cells but also by stromal cells in association with tumor development *in vivo* infers that this response is in response to novel local aspects of the tumor microenvironment. Legumain is found on the CT26 tumor cell surfaces where it is effectively removed by 30 minutes collagenase digest at 57°C (Fig. 1*B*). This further establishes the cell surface association of legumain in contrast to the previously identified intracellular localization of legumain, as there is no transmembrane domain or prior evidence of secretion or plasmalemma localization of this lysosomal protease.

To analyze the extracellular localization of legumain in tumors and normal organs, flow cytometry was used to analyze cell surface legumain in single-cell suspensions prepared from tumors, bone marrow, spleen, and kidney, as well as cultured tumor cells. Despite demonstrable intracellular legumain by renal tubular epithelial cells, <2% of isolated viable cells were very weakly positive for cell surface legumain. Spleen cells have considerably less legumain than renal cells; however, ~1% to 2% of spleen cells are weakly positive for cell surface legumain. Furthermore, cell surface legumain is not detectable on cells derived from bone marrow nor is it found on cultured CT26 cells. In contrast, 40% of intact viable cells derived from *in vivo* CT26 tumors were strongly positive for cell surface legumain (Fig. 1*C*). A similar pattern was observed for all tumors examined (data not shown), indicating that cell surface and extracellular legumain is uniquely abundant only in tumors. Using confocal microscopy analysis, we have described previously that legumain is expressed by tumor vascular endothelial cells (Fig. 2*A*;

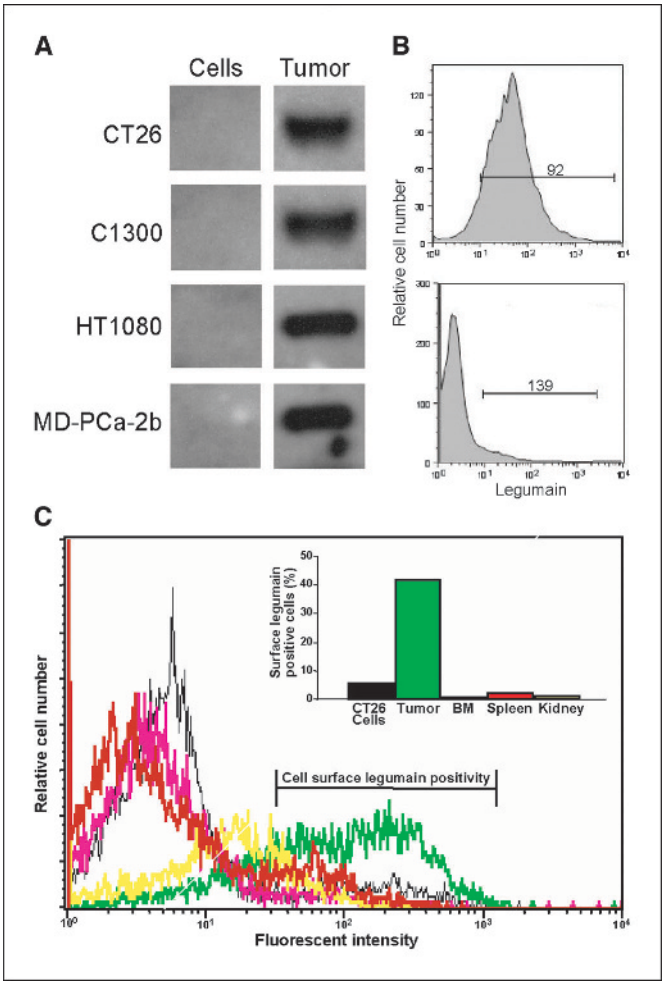

Figure 1. Legumain expression and cell surface association. *A,* Western blot analysis of cultured tumor cells and corresponding *in vivo* tumor-derived cells. *B,* flow cytometry analysis for cell surface legumain of single-cell suspensions from CT26 tumors with (*bottom*) and without (*top*) collagenase treatment. *C,* flow cytometry analysis of single-cell suspensions prepared from tumor, bone marrow (*BM*), spleen, and kidney as well as cultured CT26 tumor cells for cell surface legumain. *Columns (inset),* percentage of cells positive for surface legumain.

ref. 6). Here, we showed that legumain is expressed by tumor-associated macrophages in tissue sections from CT26 tumors by dual staining with antilegumain and anti-CD68 antibodies (Fig. 2*B*). Secreted legumain is present in the tumor stroma associating with extracellular matrix proteins, such as collagen I (Fig. 2*C*). Legumain expression is absent in normal peripheral blood monocytes. Using flow cytometry, legumain is found on the surface of viable endothelial cells and tumor-associated macrophages using both antilegumain antibody and anti-CD31 antibody or anti-CD14 antibody, respectively (Fig. 2*D*). Interestingly, legumain on endothelial cell and tumor-associated macrophage surfaces is resistant to removal by collagenase, suggesting a mode of cell surface association (Fig. 2*E*) distinct from that of tumor cells where legumain is removed by collagenase.

LEG-3 is activated by tumoral legumain. The ability of the cell-impermeable targeting tumor microenvironment prodrug LEG-3 (Fig. 3*A*) to kill tumor cells was evaluated first in cell culture (Fig. 3*B*). With both legumain transfected and control nonproducing cells, we determined the median effective concentration representing the amount of LEG-3 or doxorubicin required for 50% cell death (EC_{50}; Table 1). LEG-3 was virtually noncytotoxic to control cells not expressing legumain. However, for cells that were transfected with legumain cDNA and express cell surface legumain, cytotoxicity was significant and EC_{50} levels were close to those observed for doxorubicin, indicating an efficient conversion of the LEG-3 prodrug. The requirements for activation were shown using an alternate peptide sequence not hydrolyzed by legumain [LEG-4 (*N*-succinyl-β-alanyl-L-asparaginyl-L-leucyl-doxorubicin);

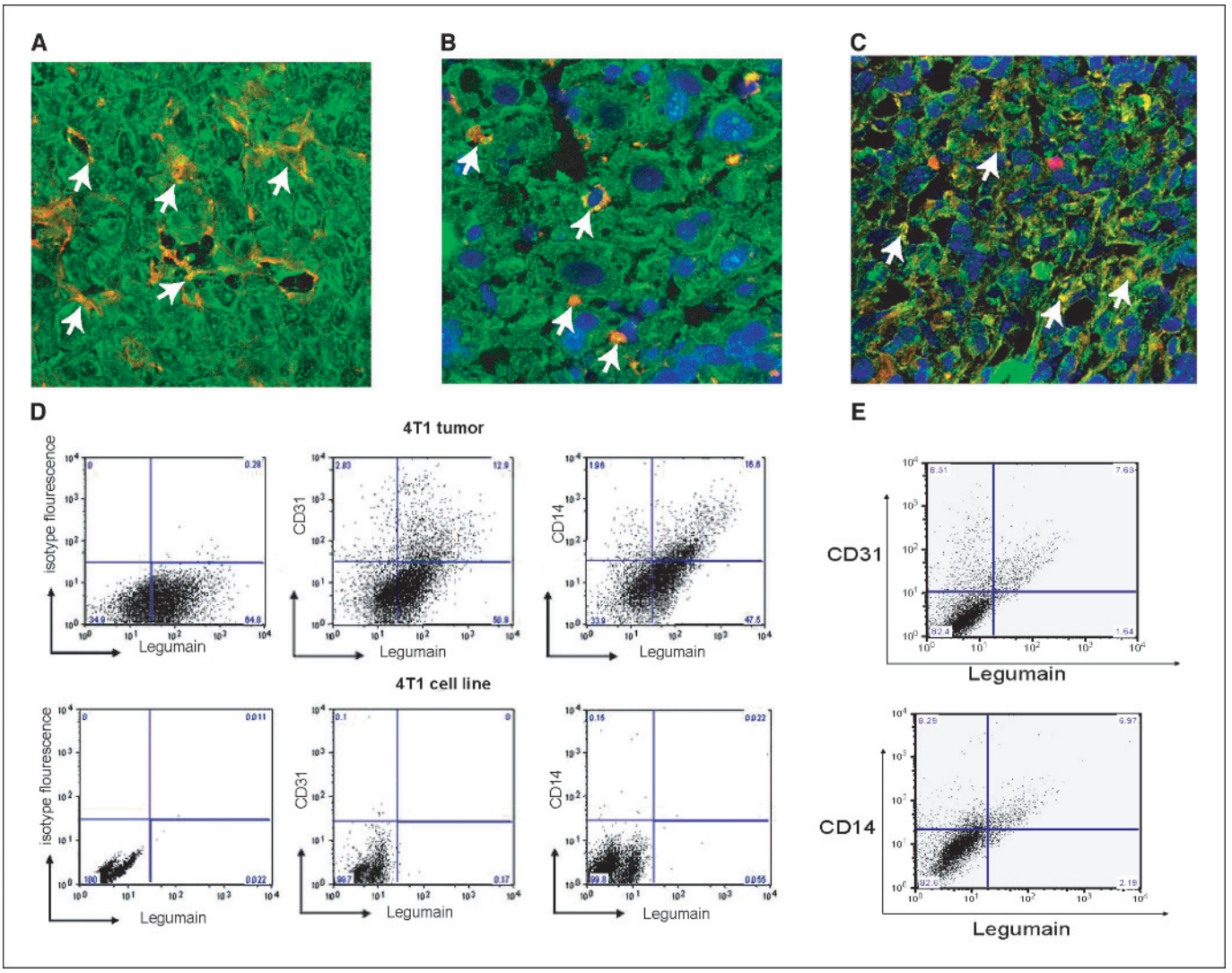

Figure 2. Legumain is expressed by stromal cells in tumors. *A,* double staining of antilegumain antibody (*green*) and anti-CD31 antibody (*red*) identifies endothelial cells. *Arrows,* tumor vascular endothelial cells expressing legumain in tumor (×400). *B,* double staining of antilegumain antibody (*green*) and anti-CD68 antibody (*red*) identifies legumain-expressing, tumor-associated macrophages (*arrows*; ×600). *Arrows,* tumor-associated macrophage cells expressing legumain. *C,* double staining of antilegumain (*green*) with anticollagen I antibody (*red*; ×400). Colocalization of legumain with collagen I in the extracellular matrix (*yellow*). *D,* two-dimensional analysis of single-cell suspensions prepared from 4T1 *in vivo* mouse mammary tumor following mechanical dissociation and of 4T1 tumor cells from tissue culture. The presence of cell surface legumain in tumor vascular endothelial cells and tumor-associated macrophages are shown with the presence of cells that are both legumain$^+$ and CD31$^+$ as well as cells that are both legumain$^+$ and CD14$^+$, respectively. *E,* two-dimensional flow cytometry of single-cell suspensions prepared from 4T1 tumors using collagenase digestion. Legumain-associated endothelial cells are represented by the group of cells that are legumain$^+$ and CD31$^+$. Legumain-associated, tumor-associated macrophages are represented by legumain$^+$ and CD14$^+$ cells.

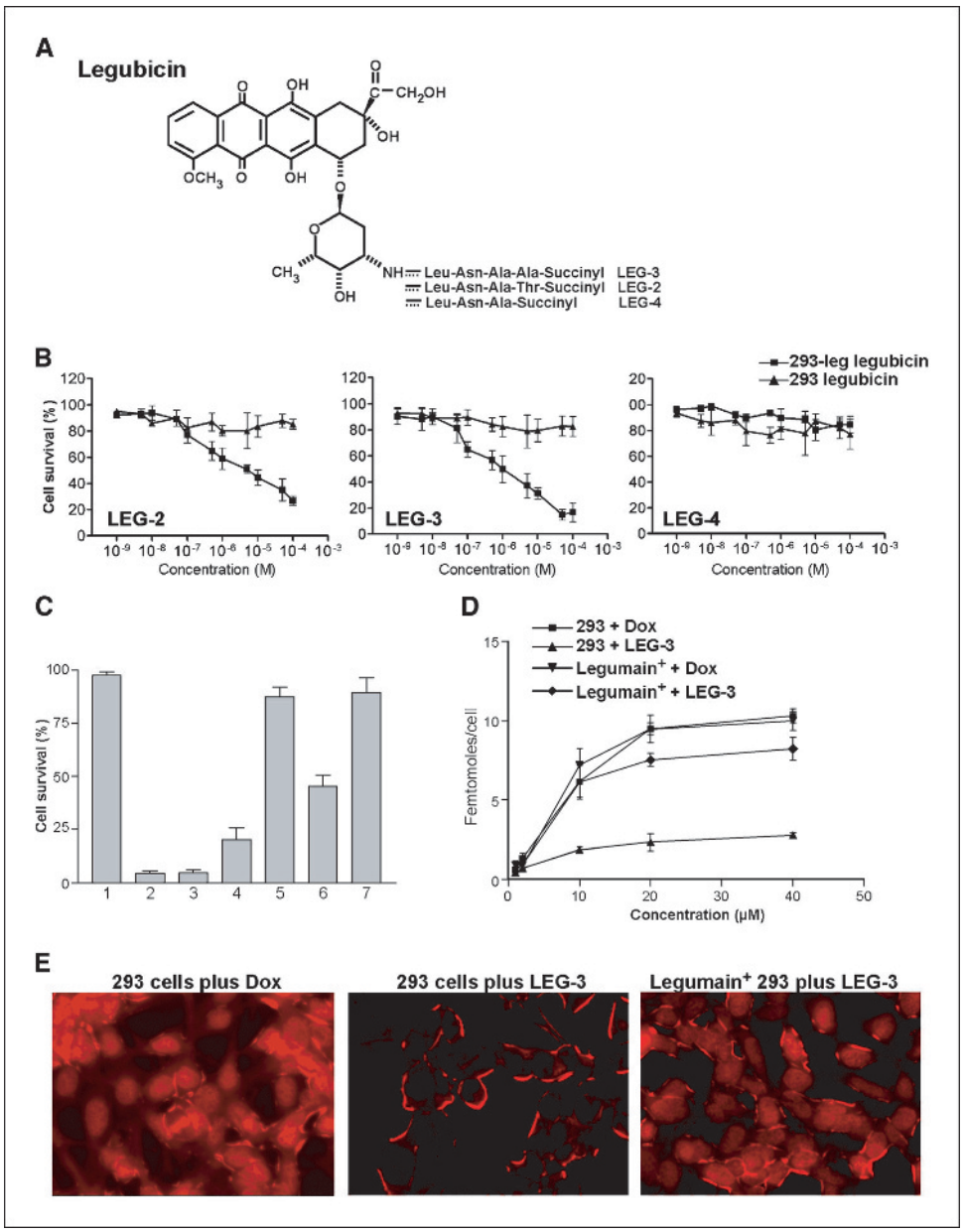

Figure 3. LEG family compounds. *A,* schematic structure of these LEGs. LEG-2 and LEG-3 are oligopeptidic derivatives of doxorubicin that are cell impermeable and can be hydrolyzed to leucine-doxorubicin by extracellular legumain. LEG-4 is similar but is not subject to legumain hydrolysis. *B,* cytotoxicity of LEG compounds using legumain$^+$ 293 cells and control wild-type 293 cells. *C,* legumain-mediated LEG activation and inhibition by cystatin. *1,* untreated control; *2,* doxorubicin treated; *3,* doxorubicin plus cystatin; *4,* LEG-3 treated; *5,* LEG-3 plus cystatin; *6,* LEG-2 treated; *7,* LEG-2 plus cystatin. *D,* cell uptake assay of LEG-3 compared with doxorubicin (*Dox*). *E,* localization of doxorubicin in cell nuclei is visualized by autofluorescence (*red*). In contrast, LEG-3 is not internalized by legumain-negative cells exposed to LEG-3 and they lack nuclear positivity for end product doxorubicin despite presence of extracellular fluorescent signal of the doxorubicin present in the LEG-3.

Fig. 3*B*] and also by inhibition of LEG-3 activation following inhibition of legumain function (Fig. 3*C*).

LEG-3 is activated extracellularly. Tumor cell uptake of LEG-3 was compared with doxorubicin. When added to cell cultures, doxorubicin rapidly entered cells. In contrast, LEG-3 remained extracellular, consistent with its observed lack of cytotoxicity. In contrast, when LEG-3 was added to legumain-expressing cells in culture, the end product doxorubicin was found in cells (Fig. 3*D* and *E*).

Legumain is selective and functional in the tumor microenvironment. When doxorubicin was administered as a single i.v. bolus, the plasma concentration very rapidly declined followed by a low concentration and slowly cleared phase, consistent with observations by other investigators (13, 14). The initial decline of infused LEG-3 was much slower, which is attributed to reduced tissue uptake (Fig. 4*A*). The content of LEG-3 in tumor tissues was determined 12 hours postinjection in mice bearing CT26 tumors. There was significant doxorubicin content in tumors, in contrast to many tissues, including heart, kidney, liver, and brain. The MTD of doxorubicin and a molar equivalent amount of LEG-3 were given i.v. For LEG-3, the amount of drug present in tumors was >10-fold greater than that for doxorubicin administration. LEG-3 was greatly reduced in cardiac tissue (Fig. 4*B*). Because of the reduced normal tissue uptake and toxicity, larger quantities of LEG-3 could be administered, which resulted in higher drug content in cells within tumors compared with that achieved for doxorubicin administration. Drug accumulation in tissues and tumor was visualized by doxorubicin autofluorescence (Fig. 4*C*). The data indicate that legumain is selectively found in the tumor microenvironment, and LEG-3 is processed to its cell-permeable Leu-doxorubicin derivative based on the presence of cytoplasmic doxorubicin, which, following processing to the end product doxorubicin, translocated to the nucleus based on intranuclear fluorescence.

***In vivo* toxicity of LEG-3 and doxorubicin.** LEG-3 is significantly less toxic than doxorubicin when evaluated *in vivo*.

Table 1. *In vitro* and *in vivo* toxicity of LEG-3 compared with doxorubicin

A. Estimated IC_{50} (μmol/L) of doxorubicin and LEG-3

	293 cells	Legumain+293 cells	HTP cells
Dox	1.2	1.4	1.5
LEG-3	>100	3.2	9.6
LEG-3 + cystatin	>100	64.4	59.2
LEG-4	>100	>100	>100

B. Estimated MTD and LD_{50} of Dox and LEG-3 (μmol/kg) in BALB/c male mice

	Dox		LEG-3	
	MTD	LD_{50}	MTD	LD_{50}
I.p.	9.8	17.2	>197.4	>197.4
I.p. 5×	2.8	3.4	98.7	>197.4
I.v.	18.2	25.8	>197.4	>197.4
I.v. 5×	3.2	5.1	74.0	197.4

C. Comparison of gross toxicity of mice treated with Dox and LEG-3

Controls					
Average weight loss (%)	4				
Dox					
Dose (μmol/kg)	1.72	5.4			
Average weight loss (%)	24	28			
Death (%)	35	75			
LEG-3					
Dose (μmol/kg)	1.97	4.9	49.4	98.7	197.4
Average weight loss (%)	3	5	7	8	16
Death (%)	0	0	0	10	50

Abbreviation: Dox, doxorubicin.

When given by six repeat i.v. administrations, LEG-3 had a much higher cumulative MTD and reduced LD_{50} compared with doxorubicin (Table 1). Bone marrow and cardiac toxicity of LEG-3 was examined and compared with doxorubicin. Also, the cytotoxic effect of LEG-3 on peripheral blood leukocyte counts (WBC) was assessed and compared with that of doxorubicin. There were no reductions of total WBC counts in mice treated six times in 12 days with 4.94 μmol/kg LEG-3, and only a slight reduction observed in mice treated with a 10-fold higher dose. In contrast, there was >50% reduction of WBC counts in the group of mice receiving as little as 3.4 μmol/kg doxorubicin by the same schedule (Fig. 5*A*). LEG-3 produced little evidence of myelosuppression compared with its parent compound doxorubicin. In cardiac tissue, mice similarly treated with 49.4 μmol/kg of LEG-3 exhibited no histologic evidence of cardiac toxicity in contrast to profound cardiac myocyte vacuolization and cell death in mice treated with doxorubicin at 3.4 μmol/kg (Fig. 5*B*). Cardiac myocytes of mice treated with doxorubicin showed marked apoptosis, which was not observed for LEG-3-treated mice (Fig. 5*B*).

***In vivo* efficacy is dependent on legumain-specific activity.** To further characterize the requirements for legumain activation of LEG-3 *in vivo*, two LEG-type compounds differing in composition of the peptidyl element of the compound were evaluated. LEG-2 (*N*-succinyl-β-alanyl-L-threoinyl-L-Asparaginyl-L-Leucyl-doxorubicin) and LEG-4 were analyzed for *in vivo* efficacy in tumor-bearing mice. The LEG-4 compound is not cleaved by legumain and was devoid of tumoricidal activity (Fig. 6*A*), whereas LEG-2 and LEG-3, which are converted by legumain, showed *in vivo* tumoricidal efficacy (Fig. 6*B* and *C*).

LEG-3 has effective tumoricidal activity against diverse tumors *in vivo*. The potential therapeutic efficacy of LEG-3 was evaluated in both syngeneic rodent tumor models and human tumor xenograft models. At a well-tolerated dose (49.4 μmol/L/kg) administered six times in 12 days, LEG-3 effectively arrested growth of murine CT26 colon carcinoma in BALB/c mice and produced complete tumor eradication in some (Fig. 6*C*). A similar level of efficacy was also observed for the murine C1300 neuroblastoma in A/J mice (Fig. 6*D*) where LEG-3 treatment induced massive tumor cell death (Fig. 6*C* and *D*), tumor eradication, and marked enhancement of survival (Fig. 6*E* and *F*).

The *in vivo* efficacy of LEG-3 on human tumor xenografts in athymic *nu/nu* mice was assessed and compared with doxorubicin. Legumain protein was not detectable in either HT1080 or MDA-PCa-2b cells in culture. However, robust legumain expression was observed by immunohistochemistry for *in vivo* tumors propagated from these cells. Indeed, LEG-3 produced potent tumoricidal activity against the HT1080 fibrosarcoma, a fast-growing tumor and a model that is traditionally sensitive to doxorubicin therapy

(Fig. 7*A*). On the other hand, human prostate carcinomas are frequently resistant to doxorubicin therapy. MDA-PCa-2b prostate carcinoma, a known doxorubicin-resistant tumor (15), failed to respond to doxorubicin *in vivo*. However, administration of LEG-3 led to complete growth arrest (Fig. 7*B*). LEG-3 was effective and frequently resulted in complete tumor eradication with marked enhancement of survival of the HT1080 as well as the MDA-PCa-2b tumor-bearing mice (Fig. 7*C* and *D*). Toxicity of LEG-3 based on weight loss and mortality was negligible (Fig. 7*E*).

Discussion

This cell-impermeable targeting tumor microenvironment–activated prodrug strategy depends on targeting as well as specific catalytic function at a pH <6.8 of a quite unique endopeptidase that is selectively expressed extracellularly in the tumor microenvironment. There, it can convert an effectively designed cell-impermeable prodrug (LEG-3) to a cell-permeable prodrug (Leu-doxorubicin), which, in turn, is converted to the active tumoricidal end product doxorubicin and translocated to cell nuclei for induction of cell death. There are distinct advantages to this strategy in that tissue uptake can occur only in the tumor or comparable pathologic microenvironment. To achieve continuous growth and remodeling, the tumor microenvironment is enriched with a variety of proteases. There are nearly 500 proteases identified thus far in the human genome (16, 17), and many have been associated with the local tumor microenvironment and seem important for tumor invasion and metastasis (18, 19). Drug access to solid tumors is relatively efficient, limited only by diffusion barriers. Compounds, such as LEG-3, converted and activated in the tumor microenvironment can access and be converted by both neoplastic and associated endothelial and stromal cells to produce substantial bystander effects. Whereas most cytotoxic drugs are designed to be cell permeable, here we diminished cell permeability, increased hydrophilic properties, and increased drug solubility to minimize tissue uptake of LEG-3. This correlates with the slower clearance from the blood as well as greatly diminished tissue accumulation.

The design of an effective tumor microenvironment–activated prodrug of this type requires knowledge of the selectivity and expression of the enzyme target, including not only functional characteristics but also *in vivo* distribution under physiologic and pathologic conditions. In respect to these issues, extracellular accessible legumain represents a promising candidate target because it is the only asparaginyl endopeptidase in the mammalian genome. We discovered that legumain is highly expressed in the majority of solid tumors (6). It is a robust acidic cysteine protease, one that is overexpressed by neoplastic cells as well as intratumoral endothelial cells and macrophages. It requires for function the local acidic tumor microenvironment and is found associated with both the extracellular matrix and cell surfaces in the tumors. In normal tissues, such as kidney, legumain is present in proximal tubular epithelial cells but only as an intracellular lysosomal protein. Although not found, extracellular legumain in normal tissues would be functionally inactive at physiologic pH (5) and such protein that may escape the tumor microenvironment would be inactive for this same reason. Legumain activity in normal tissues could be inhibited by cysteine protease inhibitors, such as

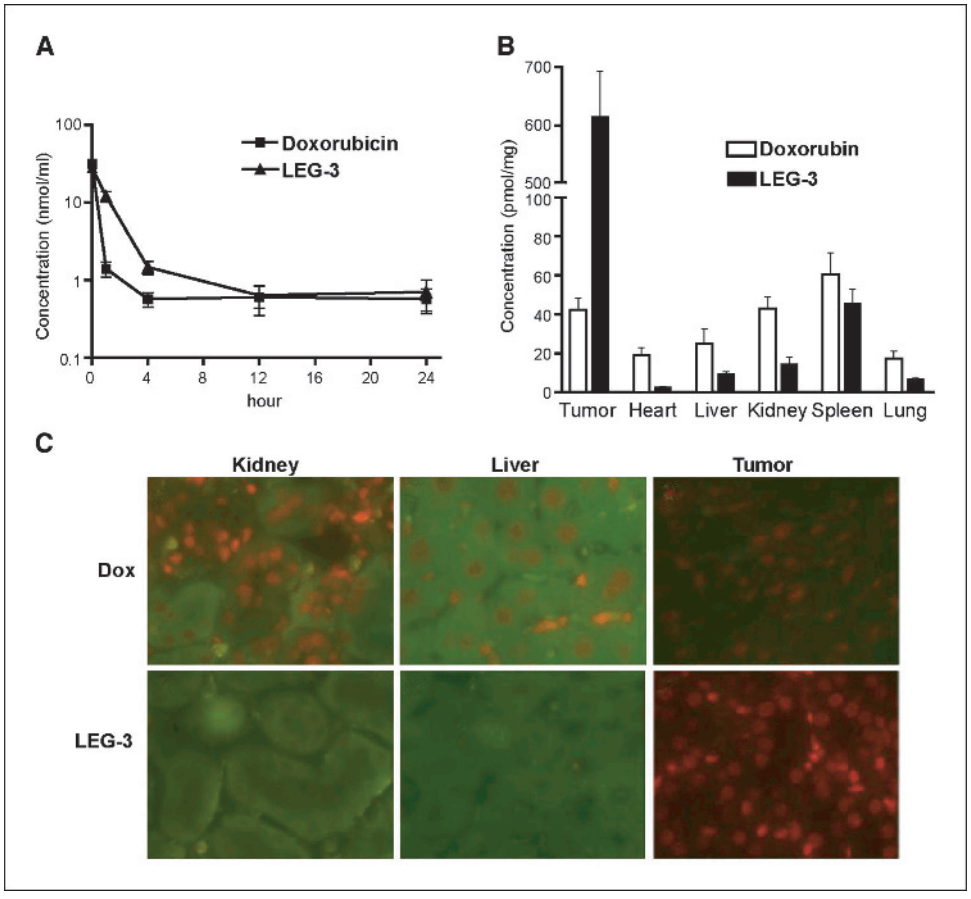

Figure 4. *In vivo* distribution and pharmacokinetics of LEG-3. *A,* plasma pharmacokinetics of LEG-3 compared with doxorubicin. *B,* accumulation of LEG-3 and doxorubicin in organs and tumors of mice bearing CT26 colon carcinomas. *C,* presence of doxorubicin and LEG-3 are visualized with autofluorescence of doxorubicin (*red*).

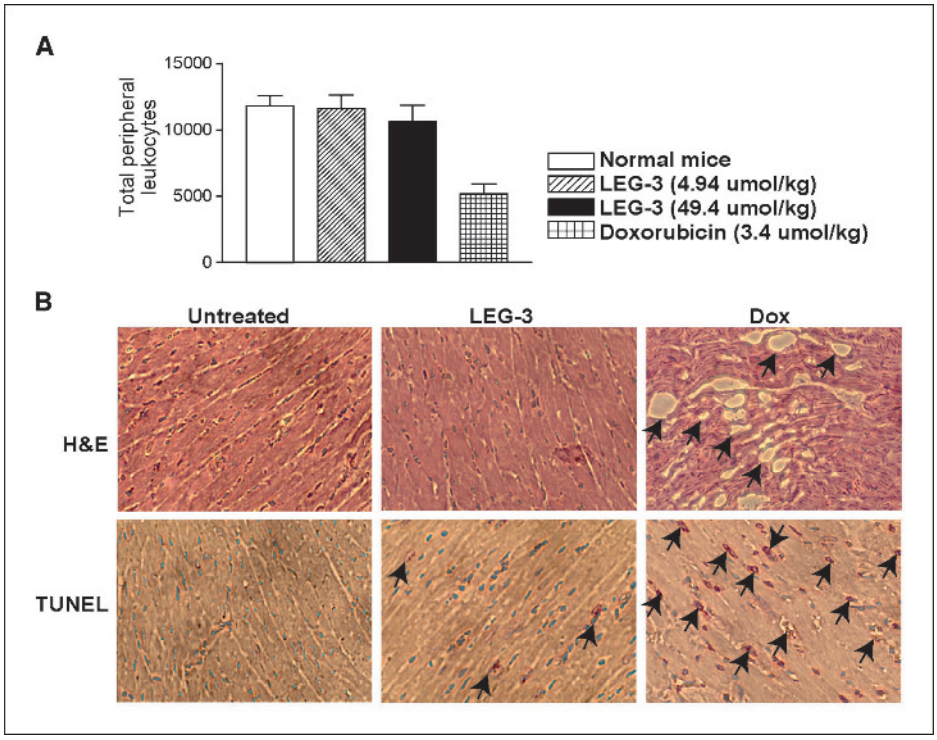

Figure 5. LEG-3 lacks *in vivo* toxicity of doxorubicin. *A,* myelosuppression of LEG-3 and doxorubicin in mice were assessed by determination of peripheral blood leukocyte counts. *B,* cardiac toxicity was shown by the presence of vacuolar degeneration of myocytes in H&E-stained sections resulting from chronic doxorubicin treatment, which was notably absent in LEG-3-treated mice. TUNEL analysis of cardiac tissue from mice treated with doxorubicin also showed marked apoptosis of myocytes (*arrows,* red apoptotic nuclei). This was very infrequent in LEG-3-treated mice.

cystatin C (20), whereas such inhibitors are commonly down-regulated in tumors (21). In addition to legumain expression, the function of reticuloendothelial system can contribute to the relative higher spleen uptake of prodrug and doxorubicin. However, we did not observe gross spleen enlargement or atrophy in prodrug-treated mice; there is no apparent cellular depletion of lymphoid or myelogenic lineages. Therefore, the legumain-activated prodrugs do not seem to have an enhanced toxicity toward spleen. The cell-impermeable targeting tumor microenvironment–activated prodrug strategy need not be restricted to doxorubicin as the cytotoxic end product. Other compounds are compatible with modification to achieve a similar tumor microenvironment–activated prodrug mode of action. Of particular interest for incorporation into this strategy are a number of highly cytotoxic compounds that have been developed based on their *in vitro* activity as exemplified by duocarmycin (22, 23).

Tumor-associated proteases have been recognized increasingly as potential targets for selective activation of prodrugs. Thus far, prodrugs were reported that are activated by prostate-specific antigen (24, 25), cathepsin (26–28), plasmin (29), and by undefined tumor-associated proteases (14, 30, 31). Here, we explored the potential of legumain to serve as a tumor-selective target and evaluated the potential of the tumor microenvironment–activated prodrug strategy. The lead candidate compound LEG-3 was analyzed *in vitro* and *in vivo.* The aminoglycoside at position C-3′ is critical for the ability of doxorubicin to integrate into DNA and to interfere with DNA Topo II and to overcome multiple drug resistance (32, 33). The addition of the legumain substrate peptidyl structure to the C-3′-NH_2 abolishes this function. It is critical to our design that charge and the succinyl cap of LEG-3 prevent cell entry. Also, the peptidyl element must not be susceptible to hydrolysis by any other enzyme. In tumor cells overexpressing legumain, LEG-3 was effectively rendered cell permeable, the resultant Leu-doxorubicin prodrug was processed with translocation of end product doxorubicin to nuclei, thereby mediating cytotoxicity. LEG-3 is effective against a variety of multidrug-resistant solid tumors, including not only rapidly progressing syngeneic rodent tumors but also slower growing human tumor xenografts in immunodeficient mice. The specific activation of LEG-3 by legumain in tumors results in higher drug delivery to tumor cells and resultant cytotoxic destruction.

Our data clearly showed that LEG-3 is a tumor microenvironment–activated prodrug that is selectively catalytically converted to end product doxorubicin in the tumor microenvironment. LEG-3 is not found in any significant amount in normal tissues presumably as a result of its cell impermeability. Based on LD_{50}, the toxicity of LEG-3 in the mouse was reduced >10-fold compared with doxorubicin. LEG-3 also exhibited a slower initial reduction in plasma concentration than doxorubicin consistent with the relative tissue impermeability. For cardiac tissue, LEG-3 accumulation was reduced >15-fold. Cardiomyopathy and the development of congestive heart failure is associated clinically with cumulative doxorubicin dosage in excess of 500 to 550 mg/m^2, a level readily achieved when required for tumors responsive to the drug, and is the major limitation for therapeutic use of doxorubicin and other anthracyclines (34). This is a notable advantage of compounds like LEG-3 because it is far less cardiotoxic. The toxicity of DNA-intercalating drugs is particularly injurious to tissues with high cell proliferation as exemplified by severe myelosuppression. We found little effect of LEG-3 on cells of myeloid lineage, as mice showed negligible reduction in peripheral blood or marrow myeloid cells at elevated therapeutic doses.

Another advantage of a tumor microenvironment–activated prodrug, such as LEG-3, over doxorubicin is the increased plasma persistence, allowing longer tumor exposure to enhance targeting. Based on the reduced toxicity, larger cumulative dosage of LEG-3 can be administered more rapidly. Consequently, significantly greater tumor inhibition and destruction have been observed for

LEG-3 in syngeneic murine colon carcinoma and neuroblastoma models without demonstrable toxicity. LEG-3 was also highly effective against human fibrosarcoma and a doxorubicin-resistant human prostate carcinoma in xenograft models where high levels of intratumoral legumain are present.

Legumain is also produced by endothelial cells and tumor-associated macrophages in the tumor microenvironment. These cells constitute additional local intratumor targets for competent drug activation and therapeutic effects, including tumor microvascular destruction. Evidence that tumor-associated macrophages can be directly tumoricidal and also stimulate tumoricidal activity of T cells is questionable. To the contrary, tumor cells frequently are able to evade the activity of tumor-associated macrophages (11). In some tumors, tumor-associated macrophages account for as many as 50% of cells. Further, evidence has emerged for a symbiotic relationship between tumor cells and tumor-associated macrophages. Tumor cells attract tumor-associated macrophages, which, in turn, provide a considerable array of growth factors and cytokines that can facilitate tumor cell survival. Tumor-associated macrophages have been reported to respond to microenvironmental factors in tumors, such as hypoxia, by producing growth factors including vascular endothelial growth factor (35–37), enzymes such

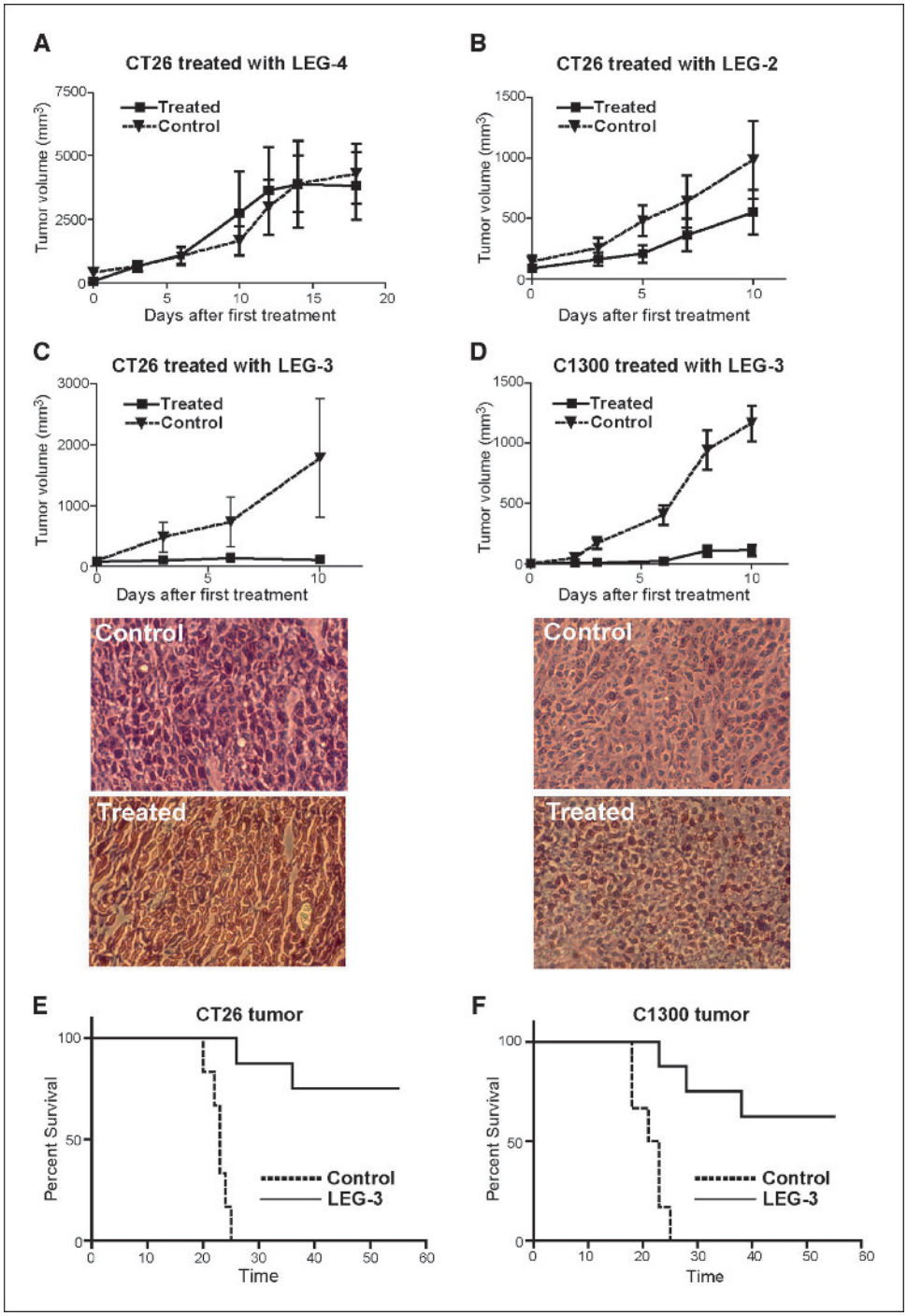

Figure 6. *In vivo* specificity and efficacy of LEG-3 in syngeneic mouse tumor models. *A, in vivo* effects of LEG-4 in mice bearing CT26 tumors (n = 8). *B,* LEG-2 (n = 8). *C,* LEG-3 (n = 8), H&E-stained sections of control versus treated tumors. *D, in vivo* effect of LEG-3 in A/J mice bearing C1300 neuroblastoma (n = 8). H&E-stained sections of control versus treated tumors. *E,* survival analysis of CT26 tumor-bearing mice treated with LEG-3. *F,* survival analysis of C1300-bearing mice treated with LEG-3.

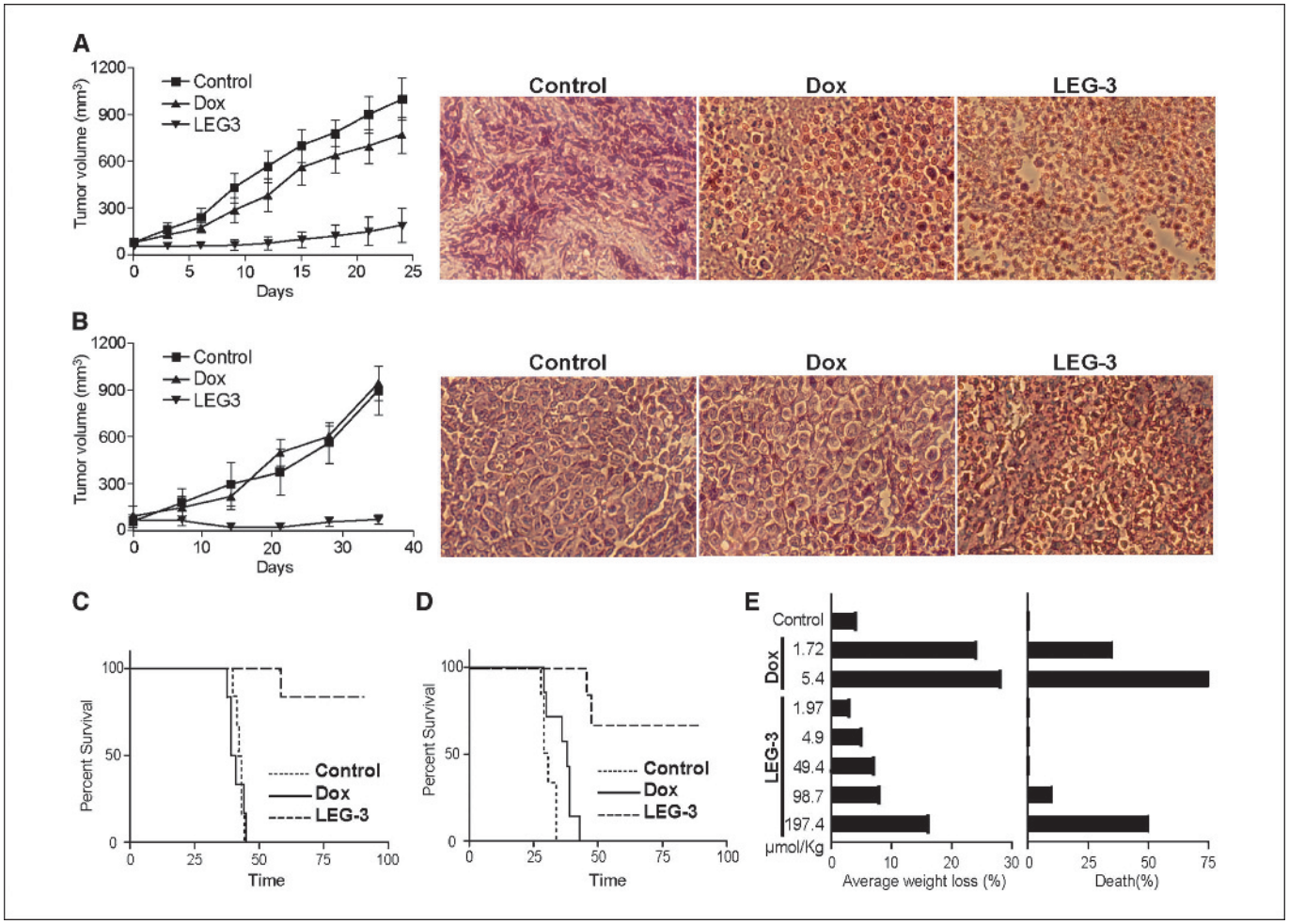

Figure 7. Efficacy of LEG-3 in human xenograft tumor models compared with doxorubicin. *A, in vivo* effects of LEG-3 (49.4 μmol/kg; $n = 8$) compared with doxorubicin (1.72 μmol/kg; $n = 8$) in the HT1080 human fibrosarcoma model and H&E-stained histologic analysis of control HT1080 tumor compared with tumors treated with doxorubicin and LEG-3. *B, in vivo* effect of LEG-3 (49.4 μmol/kg; $n = 8$) compared with doxorubicin (1.72 μmol/kg; $n = 8$) in the MDA-PCa-2b human prostate carcinoma xenograft model and H&E-stained MDA-PCa-2b human prostate carcinoma. *C,* survival analysis of HT1080 human fibrosarcoma models. *D,* survival analysis of mice bearing MDA-PCa-2b tumors after treatment. *E,* gross toxicity of tumor-bearing mice treated with doxorubicin and LEG-3.

as matrix metalloproteinases (38), cathepsins (39), and now legumain. Through these factors, tumor-associated macrophages stimulate or facilitate tumor angiogenesis, invasion, and growth, similar to the role of macrophages in wound healing. In fact, tumor cells circumvent the need to produce all the factors necessary for their growth and to establish a pseudo-organ, the solid tumor (11, 40), with the aid of tumor-derived molecules that can redirect tumor-associated macrophage activities to promote tumor survival and growth. The potent antitumor activity of LEG-3 *in vivo* may also be partly attributable to targeting endothelial cells and tumor-associated macrophages in the tumor microenvironment.

We here describe a tumor microenvironment–activated prodrug strategy for cancer therapy. LEG-3, the example, is activated by extracellular legumain in tumors leading to extensive tumor cell death and frequent complete tumor eradication, including drug-resistant tumors. These data support the advance of this strategic class of therapeutics for development as a molecularly targeted cancer therapeutic. Given that this design may be adapted to other cytotoxic compounds, it represents an opportunity to advance cancer therapy.

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A DNA Vaccine Targeting Fos-Related Antigen 1 Enhanced by IL-18 Induces Long-lived T-Cell Memory against Tumor Recurrence

Yunping Luo, He Zhou, Masato Mizutani, Noriko Mizutani, Cheng Liu, Rong Xiang, and Ralph A. Reisfeld

Department of Immunology, Scripps Research Institute, La Jolla, California

Abstract

A novel vaccination strategy induced specific CD8+ T cell–mediated immunity that eradicated spontaneous and experimental pulmonary cancer metastases in syngeneic mice and was also effective in a therapeutic setting of established breast cancer metastases. This was achieved by targeting transcription factor Fos-related antigen 1(Fra-1), overexpressed by many tumor cells, with an ubiquitinated DNA vaccine against Fra-1, coexpressing secretory IL-18. Insight into the immunologic mechanisms involved was provided by adoptive transfer of T lymphocytes from successfully immunized BALB/c mice to syngeneic severe combined immunodeficient (SCID) mice. Specifically, long-lived T memory cells were maintained dormant in nonlymphoid tissues by IL-18 in the absence of tumor antigen. Importantly, a second tumor cell challenge of these SCID mice restored both, robust tumor-specific cytotoxicity and long-lived T-cell memory, capable of eradicating established pulmonary cancer metastases, suggesting that this vaccine could be effective against tumor recurrence. (Cancer Res 2005; 65(8): 3419-27)

Introduction

The development of long-lived $CD8^+$ T-cell memory is a major goal of vaccination against tumors because it can provide continuous protection against their further dissemination and recurrence. In fact, successful protection against tumors critically depends on both, an increased number of tumor antigen–specific $CD8^+$ T cells in an immune host and the distinct capability of $CD8^+$ T memory cells to proliferate, secrete inflammatory antitumor cytokines, and repeatedly kill recurring tumor cells more effectively than naive $CD8^+$ T cells. Consequently, several ongoing efforts focus on the development of DNA-based cancer vaccines capable of inducing long-lasting immunologic memory responses endowed with specificity and a high potential to repeatedly kill tumor cells. Indeed, the establishment and continued long-term maintenance of immunologic memory has been the very key to successful tumor protective vaccination strategies (1–3).

Transcription factor Fos-related antigen 1(Fra-1), which is overexpressed by many human and mouse epithelial carcinoma cells (4–7), is involved in progression of various breast cancer cell types (8, 9) and thus represents a relevant, potential target for a breast carcinoma vaccine. Indeed, we previously showed that an oral DNA vaccine encoding murine Fra-1, coexpressing secretory murine interleukin 18 (mIL-18), induced an effective cellular immune response capable of eradicating established D2F2 breast cancer metastases in syngeneic BALB/c mice (10). IL-18 is a well-known multifunctional cytokine that was coexpressed in our DNA vaccine to enhance tumor antigen presentation by dendritic cells and to maintain an antitumor immune response. IL-18 was originally believed to elicit cytokine production by T and/or natural killer (NK) cells and to induce their proliferation and cytolytic activity, similar to an IFN-γ-inducing factor (11). The antitumor activity of IL-18 is now considered to be primarily mediated by T and NK cell activation and by enhancing cellular immune mechanisms via up-regulation of MHC class I antigen expression, favoring the differentiation of $CD4^+$ helper T cells toward the T helper 1 (Th1) subtype. In turn, Th1 cells secrete proinflammatory cytokines IL-2 and IFN-γ, which facilitate the proliferation and/or activation of $CD8^+$ CTLs, NK cells, and macrophages, all of which can contribute to tumor regression (11, 12). In addition, IL-18 is an important mediator of memory $CD8^+$ T-cell proliferation and activation via bystander activation. This process was extensively studied by Sprent et al. (13, 14), who showed that administration of innate immune activators induces proliferation of memory $CD8^+$ T cell through a mechanism involving type I IFN, IL-12, IL-15, and IL-18, respectively. Here, we extended our prior studies on a Fra-1 based DNA vaccine coexpressing IL-18 in two breast tumor models as well as a non–small cell lung carcinoma model by investigating potential working mechanisms of this vaccine, focusing particularly on the generation, function, and long-term survival of $CD8^+$ memory T cells in tumor models of syngeneic BALB/c and severe combined immunodeficient (SCID) mice after adoptive transfer of T cells from successfully vaccinated mice. We also focused on $CD8^+$ T cells that could remain dormant at high frequency in nonlymphoid tissue after successful vaccination, because their ultimate presence in the periphery is important for eliciting resistance against secondary tumor cell challenges. These questions were addressed with a polyubiquitinated DNA vaccine encoding Fra-1, cotransformed with secretory murine IL-18, and carried by attenuated *Salmonella typhimurium*, which proved capable of inducing a long-lived $CD8^+$ T-cell response that eradicated recurring D2F2 breast cancer metastases in syngeneic BALB/c mice.

Materials and Methods

Animals, bacterial strains, and cell lines. Female BALB/c and C57BL/6 mice, 6 to 8 weeks of age, were purchased from the Scripps Research Institute Rodent Breeding Facility. Female SCID mice were obtained from the Jackson Laboratory (Bar Harbor, ME). These mice were maintained under specific pathogen-free conditions and used for experiments when 7 weeks old. All animal experiments were done according to the NIH Guides for the Care and Use of Laboratory Animals. The double-attenuated *S. typhimurium* strain RE88 (*aroA*$^-$; *dam*$^-$) was obtained from Remedyne Co. (Santa Barbara, CA). The murine D2F2 breast cancer cell line was kindly provided by Dr. Wei-Zen Wei (Karmanos Cancer Institute, Detroit, MI). The murine D121 non–small cell lung carcinoma cells were a gift from Dr. L.

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Requests for reprints: Ralph A. Reisfeld, Scripps Research Institute, R218, IMM13, 10550 North Torrey Pines Road, La Jolla, CA 92037. Phone: 858-784-8109; Fax: 858-784-8110; E-mail: reisfeld@scripps.edu.

Eisenbach (Weizmann Institute of Science, Rehovot, Israel) and the murine 4T1 breast carcinoma was kindly provided by Dr. Suzanne Ostrand-Rosenberg (University of Maryland, Baltimore, MD).

Reverse transcription-PCR, Western blotting, and immunohistochemistry. *Reverse transcription-PCR (RT-PCR):* Total RNA was extracted with the Rneasy mini Kit or Rneasy tissue Kit (Qiagen, Valencia, CA) from 3 $\times$ 10^6 tumor cells of various origin: breast carcinoma cells D2F2, 4T1, and 4T07; colon carcinoma cells CT26; prostate carcinoma cells RM2; non–small cell lung carcinoma cells D121; and normal tissues from mouse spleen, liver, lung, and bone marrow. Reverse transcription was done with 1 μg of total RNA followed by PCR with the appropriate oligonucleotides. The following primers were used: ATGTACCGAGACTACGGGGAA (forward) and TCACAAAGCCAGGAGTGTAGG (reverse). The PCR was cycled 30 times at 52°C annealing temperature and quantities of RNA and PCR products were monitored for glyceraldehyde-3-phosphate dehydrogenase resulting in an 821-bp fragment. *Western blots*: Fra-1 protein expression was established in the above mentioned array of tumor cell lines and murine tissues. Western blot analysis was done with the total protein from cell lysate homogenates, using a polyclonal primary rabbit anti-murine Fra-1 antibody and anti-murine β-actin antibody as a loading control (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Specific protein was detected with a goat anti-rabbit-horseradish peroxidase (HRP)–conjugated IgG antibody (Bio-Rad, Richmond, CA). *Immunohistochemistry:* D2F2 tumor tissues were cut into sections and exposed to air until completely dry, fixed in cold acetone at −20°C for 10 minutes, and stained for Fra-1 with the DAKO immunostaining system (DAKO, Carpinteria, CA), using rabbit anti-murine Fra-1 antibody (Santa Cruz Biotechnology), diluted 1:1,000 or negative control reagents. This was followed by incubation at 4°C overnight. After three washes with PBS, a HRP-conjugated goat anti-mouse secondary antibody (DAKO) was used, and slides mounted with DAKO Faramount (DAKO). Cells were visualized microscopically, and images captured with a Nikon digital camera (Tokyo, Japan) linked to a workstation with Adobe Photoshop software (Adobe System, Inc., San Jose, CA).

Vector construction and protein expression. Two constructs were made based on the vectors pcDNA3.1/Zeo and pSecTag2/Hygro (Invitrogen, San Diego, CA), respectively. The first construct, pUb-Fra-1, was comprised of polyubiquitinated, full-length murine Fra-1. The second construct, pIL-18, contained mIL-18 with an Igκ leader sequence for secretion purposes. The empty vector served as a control. Protein expression of Fra-1 and IL-18 were shown by Western blotting with a polyclonal rabbit anti-murine Fra-1 antibody (Santa Cruz Biotechnology) and a monoclonal anti-mouse IL-18 antibody (R&D Systems, Minneapolis, MN), respectively. IL-18 protein was detected in both cell lysates and culture supernatants.

Transformation and expression of *Salmonella typhimurium* with DNA vaccine plasmids. Attenuated *S. typhimurium* (dam^-; $aroA^-$) were transduced with DNA vaccine plasmids by electroporation. Briefly, a single colony of bacteria was inoculated into 3 mL of Luria-Bertani medium, harvested during mid-log growth phase, and washed twice with ice-cold water. Freshly prepared bacteria (1×10^8) were mixed with plasmid DNA (2 μg) on ice in a 0.2-cm cuvette and electroporated at 2.5 kV, 25 μF, and 200 Ω. The bacteria were transformed with the following plasmids: empty vector, pUb-Fra-1, pIL-18, or both pUb-Fra-1 and pIL-18 combined, indicated as pUb-Fra-1/pIL-18. After electroporation, resistant colonies harboring the DNA vaccine gene(s) were cultured and stored at −70°C after confirmation of their coding sequence.

Immunization and tumor cell challenge. *Prophylactic model*: BALB/c mice or C57BL/6 mice were divided into four experimental groups ($n = 8$) and immunized thrice at 2-week intervals by gavage with 100 μL 4.5% sodium bicarbonate containing 1×10^8 doubly mutated *S. typhimurium* harboring either empty vector, pUb-Fra-1, pIL-18, or pUb-Fra-1/pIL-18. All mice were challenged by i.v. injection with 5×10^5 D2F2 cells (BALB/c) or 2×10^5 D121 cells (C57BL/6) or fat pad injection with 7×10^3 4T1 cells (BALB/c), 1 week after the last immunization, to induce either experimental or spontaneous pulmonary metastases. The survival rate of mice, lung weight, and metastatic scores in experimental or control groups were observed. Two months after the first tumor cell challenge, the surviving mice from the treatment groups in only the D2F2 model were divided into two groups; one was reimmunized with pUb-Fra-1/pIL-18 and the other group received no reimmunization. All mice were rechallenged with 0.5×10^6 D2F2 cells 1 week after reimmunization and sacrificed at either 2 or 8 weeks after the first challenge or 1 week after rechallenge with tumor cells to determine the proliferation of $CD8^+$ T cells and IFN-γ release. *Therapeutic model:* BALB/c mice were divided into four experimental groups ($n = 8$) and injected i.v. with 0.5×10^6 D2F2 cells on day 0 and immunized with DNA vaccine thrice as described above. The experiment was terminated on day 28 to observe mouse lung weights.

Adoptive transfer of lymphocytes. BALB/c mice that served as donors of tumor-specific $CD8^+$ T cells for adoptive transfer experiments were those animals who were previously successfully immunized thrice at 2-week intervals with the pUb-Fra-1/pIL-18 vaccine. These animals were then challenged with 0.5×10^6 D2F2 tumor cells i.v. 1 week later and remained tumor free 2 months thereafter. Mice comprising the control group were immunized only with empty vector. The animals in all treatment groups were sacrificed 2 days after being reimmunized with the pUb-Fra-1/pIL-18 vaccine or after receiving no reimmunization. Lymphocytes were harvested via cannulation of the spleen and separated by Ficoll/hypaque gradient centrifugation ($600 \times g$, 20 minutes). Naive syngeneic SCID mice were reconstituted with a total of 4.5×10^7 lymphocytes by i.v. injection of 1.5×10^7 lymphocytes each, on days 0, 2, and 4. Their fate was then followed by flow cytometric analyses on days 7, 14, and 30, with anti-CD8 and anti-CD3 antibodies, respectively. After 2 days, individual groups of mice were challenged by i.v. injection of 0.5×10^6 D2F2 cells to initiate experimental pulmonary metastases. Tumor specific cytotoxicity and IFN-γ release, were determined 3, 7, and 30 days after tumor cell challenge and the survival rate of these SCID mice was observed.

Preparation of lymphocytes from nonlymphoid tissue. The lung vascular bed was flushed with 10 mL chilled HBSS (Life Technologies, Gaithersburg, MD) introduced via cannulation of the right ventricle. Lymphocytes were incubated for 60 minutes at 37°C in a solution of enzymes (i.e., 125 units/mL collagenase, Life Technologies; 60 units/mL each of Dnase I; and 60 units/mL of hyaluronidase, Sigma, St Louis MO). The cell suspension was layered over a lymphocyte-M (Cedarlane Laboratories, Hornby, Canada) density gradient (15), centrifuged at $600 \times g$ for 20 minutes at 25°C, and lymphocytes washed two to three times before further processing. Blood was flushed from livers by injecting 5 mL of RPMI 1640 through the portal vein. Leukocytes from the liver were then isolated by crushing this organ in a tissue grinder followed by incubation with the above enzyme solution and collection of the leukocyte layer from a Metrizamide (Sigma-Aldrich, St. Louis, MO) density gradient. Contaminating erythrocytes were removed from the leukocyte preparations by treatment with ACK lysis buffer (Cambrex Bio Science Walkersville, Inc., Walkersville, MD).

***In vitro* depletion of $CD4^+$ or $CD8^+$ T cells.** The depletion of $CD4^+$ or $CD8^+$ T cell *in vitro* was done as previously described (16). Briefly, splenocytes were isolated from C57BL/6 mice after vaccinations with experimental or control DNA vaccines, 2 weeks after challenge with D121 tumor cells. $CD4^+$ T-cell depletion was accomplished with 10 μg/10^7 splenocytes of anti-CD4 (derived from hybridoma GK1.5), and $CD8^+$ T cells were depleted with anti-CD8 antibody (derived from hybridoma 2.43) for 30 minutes at 37°C. Rabbit serum complement (1:6) was added with 1 mL diluted complement to 10^7 cells/mL. Cells were incubated for 30 minutes at 37°C, washed, and resuspended for the CTL assay. All antibodies were purchased from National Cell Culture Center (Minneapolis, MN). Rabbit serum complement was obtained from Serotec, Inc. (Raleigh, NC).

Cytotoxicity assay. Cytotoxicity was measured and calculated by a standard ^{51}Cr-release assay. Briefly, in the D2F2 tumor model splenocytes were harvested from BALB/c SCID mice at 3, 7, and 30 days after challenge with 5×10^5 D2F2 breast carcinoma cells after passive transfer of lymphocytes. In the D121 lung tumor model, splenocytes were obtained from C57BL/6 mice 2 weeks after challenge with 2×10^5 D121 tumor cells following either $CD4^+$ or $CD8^+$ T-cell depletion *in vitro*. These cells were then stimulated *in vitro* by irradiated (1,000 Gy) D2F2 cells or D121 cells for 4 days at 37°C in complete T-STIM culture medium (Becton Dickinson, Bedford, MA) containing 10% fetal bovine serum and recombinant IL-2 at 20 units/mL (PeproTech, Rocky Hill, NJ). These D2F2 or D121 target cells were then labeled with ^{51}Cr for 2 hours at 37°C and incubated with effector cells at

various effector-to-target cell ratios at 37°C for 4 hours. The percentage of specific target cell lysis was calculated by the formula $[(E - S) / (T - S)] \times 100$, where E is the average experimental release, S the average spontaneous release, and T the average total release.

Flow cytometry. Activation markers of T cells were measured by two-color flow cytometric analysis with a BD Biosciences FACScalibur. T-cell markers were determined by staining freshly isolated lymphocytes from successfully vaccinated mice or from passively transferred SCID mice with anti-CD8 antibodies in combination with FITC-conjugated anti-CD3 antibody. Memory $CD8^+$ T cells bearing high levels of $CD44^{high}$ and $CD122^+$ (IL-2Rβ) were quantified by three-color flow analysis. Splenocytes were isolated from successfully vaccinated BALB/c mice or from passively transferred SCID mice and then stained with anti-CD8-Cy-Chrome, anti-CD122-PE and anti-CD44-FITS antibodies, followed by fluorescence-activated cell sorting (FACS) analyses. All antibodies were purchased from PharMingen (San Diego, CA). IL-2 release at the intracellular level was determined in lymphocytes of Peyer's Patches obtained 3 days after one time immunization and stained with APC-anti-CD4 or CD8 and combined with FITC-anti-CD69. Cells were fixed, permeabilized, and subsequently stained with PE-labeled anti-IL-2 antibodies to detect the intracellular expression of IL-2.

ELISPOT assay. ELISPOT assays were done to measure single cell release of IFN-γ. Splenocytes were collected 2 weeks after D121 tumor cell challenge from all experimental groups of C57BL/6 mice or 2, 7, and 30 days after lymphocyte transfer to SCID mice (only in the D2F2 tumor model), and splenocytes from control mice immunized only with the empty vector. After lysis of RBC with ACK lysis buffer, splenocytes were resuspended at a final concentration of 1×10^7/mL (D121 tumor model) or 2×10^6/mL (D2F2 tumor model), and 100 μL of this suspension was cultured for 24 hours in complete T-cell medium with or without 100 μL irradiated (1,000 Gy) D121 cells (1×10^5/mL) or D2F2 cells (1×10^4/mL). The assay was done according to instructions provided by the manufacturer (BD Bioscience, San Jose, CA). Plates were read by immunospot ScAnalysis and digitalized images were analyzed for areas in which color density exceeded background by an amount based on a comparison with experimental wells.

Statistical analysis. The statistical significance of differential findings between experimental groups and controls was determined by Student's t test. Findings were regarded as significant if two-tailed Ps < 0.05.

Results

Differential expression of Fra-1 in tumor cell lines and normal mouse tissues. To study the distribution and expression of the Fra-1 antigen in mouse tumor models, we examined its differential expression in normal and mouse tumor tissues by analyzing expression of mRNA with RT-PCR in normal tissues of spleen, liver, lungs, and bone marrow and in breast tumor cell lines D2F2, 4T1, 4T07, prostate carcinoma RM2, non–small cell lung carcinoma D121, and CT26 colon carcinoma cells. Expression of mRNA levels of Fra-1 was markedly increased in all of these tumor cell lines but was detectable only at very low levels in all normal tissues (Fig. 1*A*). This differential expression of Fra-1 was confirmed at the protein level by Western blotting, revealing high expression in D2F2 cells and somewhat lower expressions in RM2 and CT-26 cells. In contrast, Fra-1 protein level was uniformly expressed at very much lower levels in all of the normal murine tissues examined (Fig. 1*B*). Furthermore, immunohistochemical analysis indicated strong Fra-1 expression in D2F2 breast cancer tissue when paraffin-embedded sections were stained with anti-Fra-1 antibody (Fig. 1*C*, *I* and *III*) when compared with negative control sections stained without the primary anti-Fra-1 antibody (Fig. 1*C*, *II* and *IV*).

Fra-1/IL-18-based DNA vaccine induces effective antitumor immunity. We proved our hypothesis that an orally given DNA vaccine encoding murine Ub-Fra-1 and secretory IL-18, carried by attenuated *S. typhimurium*, can induce an effective antitumor immune response. We found an increase in life span of BALB/c mice (n = 8) vaccinated as described above and challenged 2 weeks later by i.v. injection of a lethal dose (5×10^5/mL) of D2F2 breast carcinoma cells. The life span of 62% of successfully vaccinated BALB/c mice (5 of 8) tripled in the absence of any detectable tumor growth up to 98 days after tumor cell challenge (Fig. 2*A*, *a*).

Vaccination reduces growth of established metastases. Marked inhibition of growth of established metastases was observed in C57BL/6 mice challenged by i.v. injection of D121 non–small cell lung carcinoma cells 2 weeks after the third vaccination with the Fra-1/IL-18-based vaccine as described above. In contrast, animals vaccinated with only the empty vector carried by the attenuated bacteria, revealed uniformly rapid metastatic pulmonary tumor growth of D121 non–small cell lung carcinoma (Fig. 2*A*, *b*). Our vaccine was also effective in a therapeutic setting. This was shown by an initial i.v. injection of BALB/c mice (n = 8) with D2F2 breast carcinoma cells and vaccination of these mice 5 days thereafter with our Fra-1/IL-18 vaccine when these mice had established pulmonary metastases, and by collecting their lungs 28 days later. All such treated mice showed lower lung weights and markedly reduced tumor burden, whereas all control animals treated with the empty vector revealed much increased lung weights and tumor burden (Fig. 2*B*).

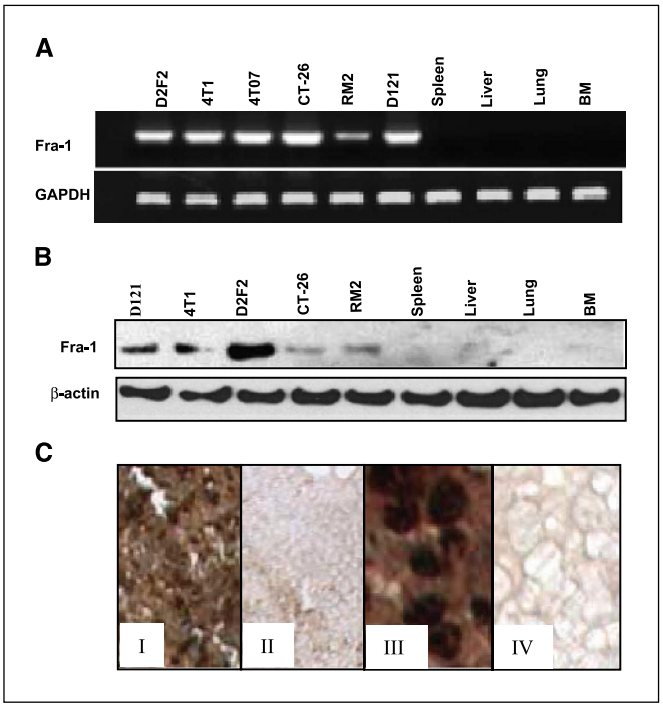
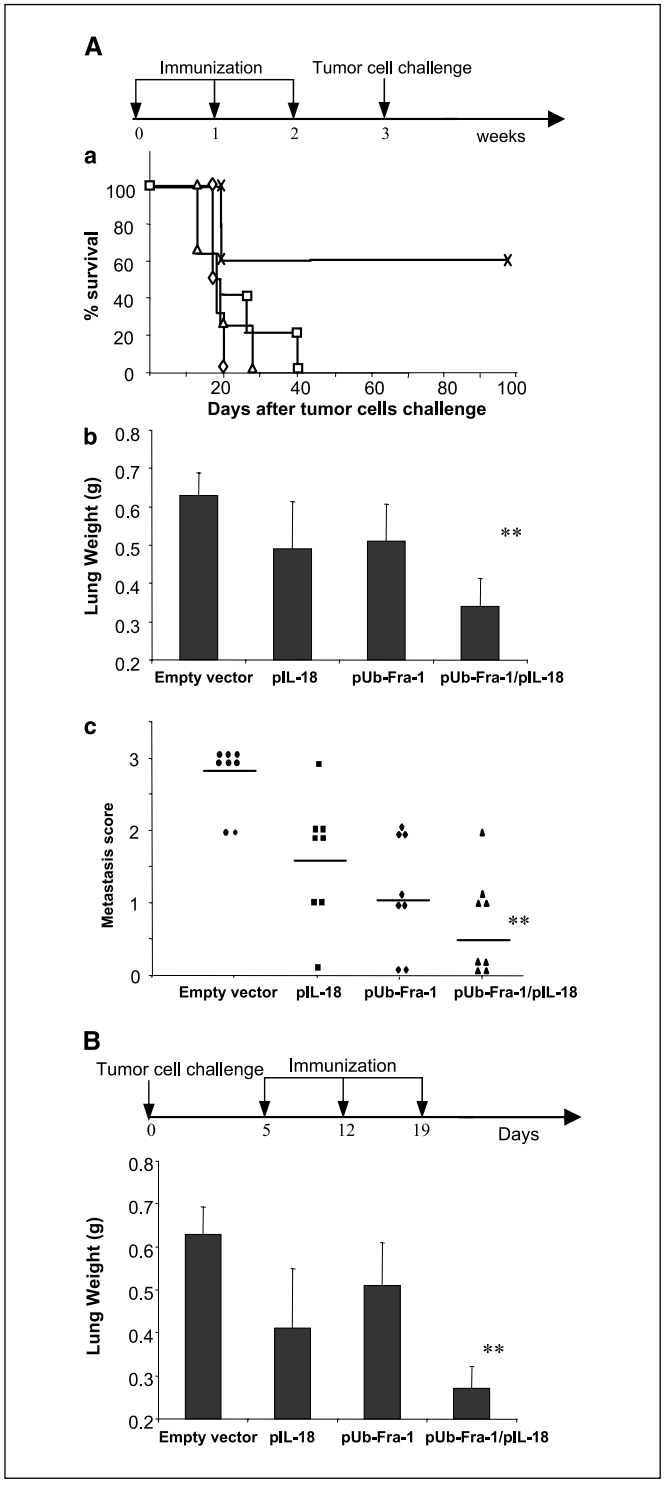


Figure 1. Expression of the murine Fra-1 molecule in normal mouse tissues and tumor cell lines. *A,* RT-PCR analysis of Fra-1 gene expression by carcinoma cell lines D2F2, 4T1, 4T07 (breast), CT-26 (colon), RM2 (prostate), and D121 (non–small cell lung) as well as normal murine tissues from spleen, liver, lungs, and bone marrow. Total RNA was extracted from cells growing at 70% confluence and from normal murine tissues. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a control for total RNA loading. *B,* Western blot analysis of Fra-1 protein expression in the above mentioned tumor cell lines and normal mouse tissues. Protein lysates were extracted from cells growing at 80% confluence. Homogenized normal tissues and β-actin were used as controls for protein loading. *C,* immunohistochemical analysis of Fra-1 in D2F2 breast cancer tissue. Paraffin-embedded sections from D2F2 breast cancer tissue samples were analyzed by immunohistochemistry using antibody against Fra-1 protein. Immunohistochemical staining of D2F2 breast cancer tissue with anti-Fra-1 antibody (*I*, ×10 magnification; *III*, ×40 magnification) and without using primary anti-Fra-1 antibody (*II*, ×10 magnification; *IV*, ×40 magnification).

Protection against spontaneous pulmonary metastases. We noted a marked reduction in dissemination of spontaneous pulmonary metastases of 4T1 breast carcinoma cells after three immunizations with the Fra-1/IL-18-based DNA vaccine. This became evident 28 days after surgical excision of fat pads bearing primary 4T1 breast carcinoma and as confirmed by visual examination of the lungs of these animals for metastases, which established their metastatic score (Fig. 2*A*, *c*).



CD8⁺ T cells are responsible for the antitumor response. Evidence for an activated T-cell immune response was indicated by three lines of evidence. First, we found that only the vaccine encoding pUb-Fra-1/pIL-18 proved highly effective in markedly up-regulating IL-2 expression on $CD69^+$, $CD4^+$, or $CD8^+$ activated T cells during T-cell priming (Fig. 3*A*). Second, only lymphocytes isolated from mice immunized with this vaccine were effective in specifically killing D121 non-small cell lung cancer cells *in vitro* at different effector-to-target cell ratios. In contrast, lymphocytes isolated from vaccinated mice that were thereafter depleted of $CD8^+$ T cells *in vitro* failed to induce cytotoxic killing of D121 tumor target cells. However, *in vitro* depletion of $CD4^+$ T cells did not abrogate cytotoxic killing of these same tumor target cells (Fig. 3*B*). The same results were obtained in the D2F2 breast carcinoma model in BALB/c mice (data not shown). Third, release of IFN-γ from T cells, a well-known indication of T-cell activation in secondary lymphoid tissues, was found at the single cell level by ELISPOT (Fig. 3*C*) only after vaccination with the pUb-Fra-1/pIL-18 plasmid. In fact, subsequent challenge with tumor cells induced a dramatic increase in IFN-γ release when compared with that of splenocytes from control mice. Taken together, these data suggest that the activation of T cells was specific for Fra-1.

Activation of specific T cells in lymphoid tissue is followed by migration to nonlymphoid tissue. Interactions between IL-18 and active Th1 cells are believed to be critical for achieving both, optimal antigen-specific T-cell responses in lymphoid tissues and activated T-cell migration to nonlymphoid tissues in the local tumor microenvironment. To prove this contention in our models, we analyzed $CD8^+$ T cells in both lymphoid and nonlymphoid tissues. The vaccine encoding pUb-Fra-1/pIL-18 substantially up-regulated the $CD8^+$ T-cell populations 2 weeks after challenge with D2F2 tumor cells in both lymphoid tissues (spleen) as well as in blood and nonlymphoid tissues, especially in local lung tumor tissues (Fig. 4*A*, *I*). Eight weeks after tumor-cell challenge, the level of $CD8^+$ T cells in all these tissues declined and, in fact, this decline was more rapid in lymphoid tissues (spleen) than in nonlymphoid tissues (lungs; Fig. 4*A*, *II*). However, when 8 weeks after the initial tumor cell challenge, all surviving mice were rechallenged with D2F2 cells, $CD8^+$ T-cell populations were again dramatically up-regulated and these same

Figure 2. Effect of protective immunity induced by the pUb-Fra-1/pIL-18-based DNA vaccine on different tumor metastases models. *A,* prophylactic model. Vaccination schedule designed for three immunizations at 2-week intervals, followed by i.v. challenge with 0.5×10^6 D2F2 or 0.2×10^6 D121 tumor cells or fat pad injection with 0.7×10^4 4T1 tumor cells 1 week after the last immunization. Kaplan-Meyer plot (*a*) of the survival of eight mice in each of the treatment regimens (□) pUb-Fra-1, (△) pIL-18, (×) pUb-Fra-1/pIL-18, and (◇) control groups. Surviving mice were tumor free unless otherwise stated. Representative lung specimens of C57BL/6 mice ($n = 8$) were obtained 4 weeks after challenge with D121 non–small cell lung carcinoma cells (*b*). *Columns,* average lung weight (g) in each group. Normal lung weight is ~0.2g. **, $P < 0.001$, $P < 0.05$, $P < 0.05$ compared with empty vector, pIL-18, or pUb-Fra-1, respectively. Experiments were repeated three times with similar result. Representative lung specimens from BALB/c mice ($n = 8$) challenged with 4T1 breast carcinoma cells by fat pad injection 4 weeks after removal of primary tumor (*b*). Tumor metastasis scores on lungs were established by estimating the % surface area covered by metastases as follows: 0, no metastases; 1, <20%; 2, 20% to 50%; and 3, >50% represented by individual symbols for each treatment group. *Short lines,* average metastases score of each group. **, $P < 0.001$, $P < 0.05$, $P < 0.05$ compared with empty vector, pIL-18, or pUb-Fra-1, respectively. *B,* therapeutic model. Groups of BALB/c mice ($n = 8$) were initially injected i.v. with 0.2×10^6 D2F2 cells on day 0 and vaccinated with the DNA vaccine on days 5, 12, and 19, respectively. The experiment was terminated on day 28. *Columns,* average lung weight (g) in each group. **, $P < 0.001$, $P < 0.05$, and $P < 0.05$ compared with empty vector, pIL-18, or pUb-Fra-1, respectively.

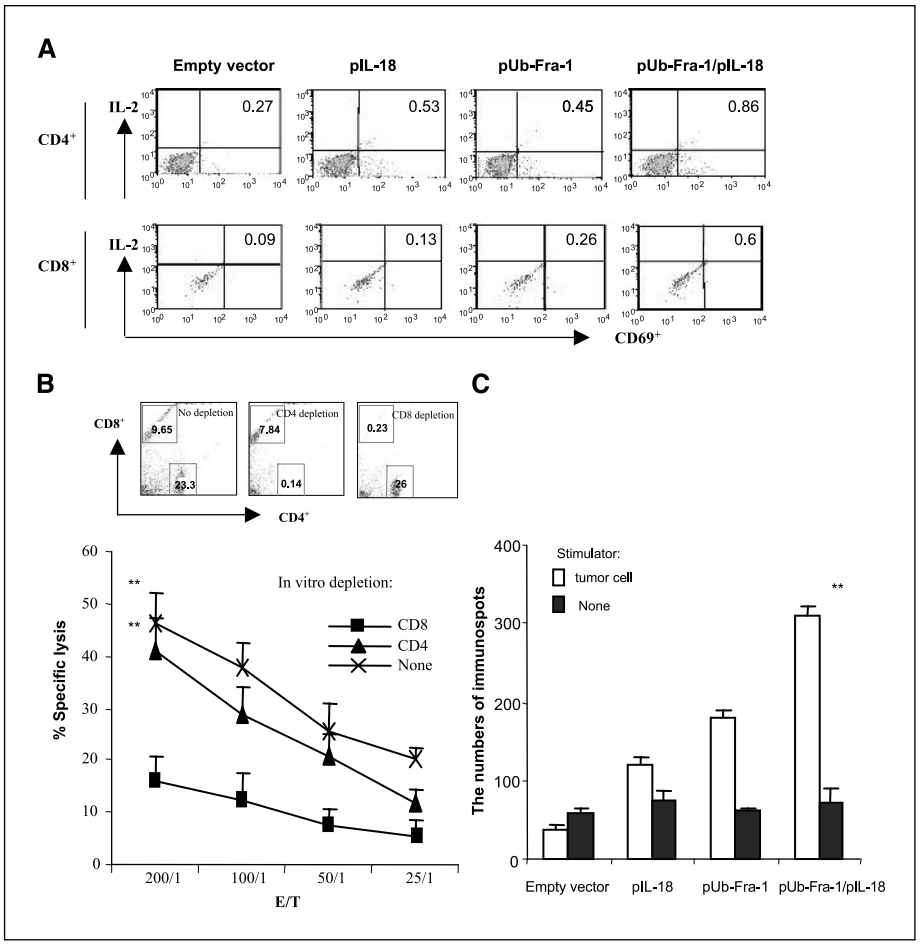


Figure 3. T-cell activation by the pUb-Fra-1/pIL-18-based vaccine in the non–small cell lung carcinoma model. *A,* up-regulated IL-2 expression of primed activated T cells. Three-color flow cytometric intracellular staining analyses were performed with single-cell suspensions of lymphocytes of Peyer's Patches obtained from immunized mice 3 days after one immunization. Cells were stained with APC-labeled anti-$CD8^+$ or anti-$CD4^+$ antibodies, FITC-labeled anti-$CD69^+$ antibodies, and PE-labeled anti-IL-2 antibodies and analyzed and gated on live $CD8^+$ or $CD4^+$ T cells. *B,* induction of $CD8^+$ T cell–specific cytotoxic activity. Splenocytes were isolated from C57BL/6 mice after vaccination with either experimental or control DNA vaccines 2 weeks after challenge with D121 tumor cells and analyzed for their cytotoxic activity in a ^{51}Cr-release assay at different effector-to target cell ratios. Specific lysis is shown mediated by $CD8^+$ T cell against D121 tumor target cells Depicted are cytotoxicity without depletion (×), specific lysis following depletion of $CD8^+$ T cell (■) or of $CD4^+$ T cells (▲). *Point,* mean of eight animals. **, $P < 0.001$ compared with no T-cell depletion. *C,* production of IFN-γ. This is indicated at the single T cell level either without (□) or with stimulation (■) as determined by the ELISPOT assay and depicted by the number of immunospots formed per well. Mean spot distribution of each well in each experimental and control group ($n = 4$, mean ± SD). **, $P < 0.001$, $P < 0.01$, and $P < 0.01$ compared with empty vector, pIL-18, or pUb-Fra-1, respectively.

cells increased even more rapidly in lung tissue than in lymphoid tissues (Fig. 4*A*, *III*). Furthermore, ELISPOT assay indicated IFN-γ release from these same lymphocytes and T-cell activation was confirmed by release of proinflammatory cytokine IFN-γ which increased in both lymphoid and nonlymphoid tissues 2 weeks after the first tumor cell challenge (Fig. 4*B*, *I*). However, 8 weeks after the initial tumor cell challenge, IFN-γ release decreased (Fig. 4*A*, *II*) but then increased again dramatically after rechallenge with D2F2 tumor cells, especially in lung lymphocytes (Fig. 4*A*, *III*).

A specific memory T-cell response is induced and maintained in the absence of tumor antigen. We tested the hypothesis that $CD8^+$ T cells, adoptively transferred from successfully immunized mice to syngeneic SCID mice, and parked there for 7 or 30 days, could maintain effective and long-lived memory in the absence of both tumor antigen and naive T cells. To this end, SCID mice were adoptively transferred with lymphocytes that were harvested from successfully immunized mice that had remained tumor free for at least 98 days after the initial tumor cell challenge and were then subjected to either reimmunization with the same DNA vaccine or to no reimmunization. The data depicted in Fig. 5*A* indicate that the life span of 75% (6 of 8) in the reimmunized group of mice and in 62% (5 of 8) in the nonreimmunized group of SCID mice was tripled in the absence of any detectable tumor growth up to 56 days after tumor cell challenge. Importantly, the continuous presence of tumor antigen was not required to maintain long-lived $CD8^+$ T-cell memory among $CD8^+$ T cells that were adoptively transferred into syngeneic SCID mice (Fig. 5*A*). Furthermore, we determined the fate of $CD8^+$ T effector cells in the absence of tumor antigen by adoptive transfer of lymphocytes from immunized BALB/c mice into SCID mice. Thus, when these animals' splenocytes were subjected to FACS analysis to detect the presence of $CD8^+$ T cells, there was a continuous decrease in the number of these cells for 30 days suggesting that the majority of these T effector cells gradually apoptosed (Fig. 5*B*).

Rapid turnover of memory $CD8^+$ T cells after vaccination and repeated tumor cell challenges. We determined that vaccination with the pUb-Fra-1/pIL-18 construct followed 2 weeks thereafter by a D2F2 tumor cell challenge, leads to a rapid turnover of $CD8^+$ T memory cells. This was indicated by up-regulated expression of $CD8^+$, $CD44^{high}$, $CD122^+$ memory T-cell markers at different time points in both lymphoid and nonlymphoid tissues (Fig. 6*A*). Thus, turnover of these memory T cells occurred just 24 hours after tumor cell challenge and reached a peak at 72 hours (Fig. 6*B*). Importantly, we could also verify that these putative, specific $CD8^+$ memory T cells can also effectively recognize a second challenge of D2F2 tumor cells. If fact, we not only detected increased expression of $CD8^+$, $CD44^{high}$, $CD122^+$ memory T cells in both lymphoid and nonlymphoid tissues 56 days after the first tumor cell challenge in syngeneic BALB/c mice (Fig. 6*C*, *I*) but also found the same up-regulation of these $CD8^+$ memory T-cell markers when these very same lymphocytes were adoptively transferred to SCID mice that were subjected 1 week thereafter to a D2F2 tumor cell challenge (Fig. 6*C*, *II*). Moreover, the $CD8^+$, $CD44^{high}$, $CD122^+$ memory T-cell expression was more pronounced

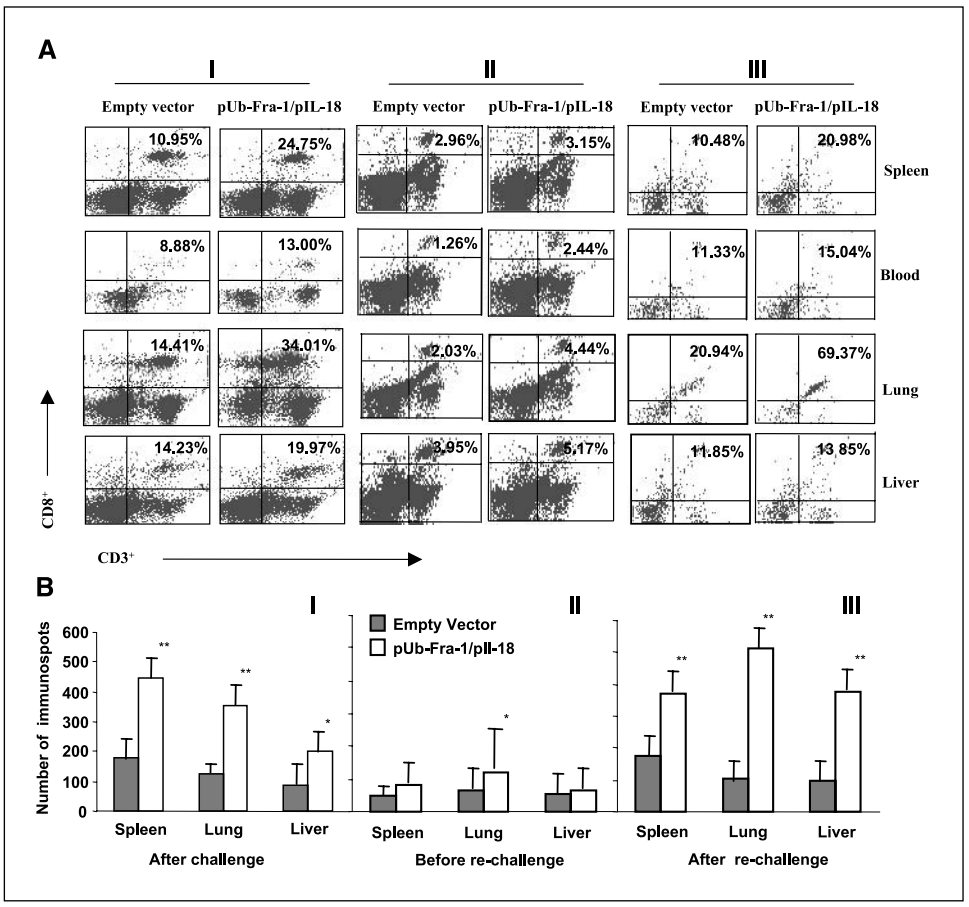


Figure 4. T cell activation in nonlymphoid tissue. *A,* up-regulated CTL markers. Lymphocytes were isolated from spleen, lungs, liver, and blood of immunized mice 2 weeks (*I*) or 8 weeks (*II*) after D2F2 (0.5×10^6) i.v. tumor cell challenge as well as 1 week after rechallenge (*III*) of mice surviving after 14 weeks. Mice treated only with empty vector served as controls. Two-color flow cytometric analyses were performed with single-cell suspensions of lymphocytes. PE-labeled anti-CD8 antibodies were used in combination with FITC-conjugated anti-mouse CD3 monoclonal antibody with each value representing the mean of four mice. Differences between the results obtained with the control group (empty vector) were statistically significant when compared to those of the treatment group (pUb-Fra-1/pIL-18). $P < 0.05$ and especially significant in the group of animals where lymphocytes were obtained from lungs ($P < 0.001$). *B,* IFN-γ release from lymphocytes in different tissues. Lymphocytes were isolated as described above. Production of IFN-γ was detected at the single T-cell level by the ELISPOT assay. *Column,* lymphocytes from four mice. Differences between the control group (empty vector) and the treatment group (pUb-Fra-1/pIL-18) were statistically significant. *, $P < 0.05$ and **, $P < 0.001$.

in local lung tumor tissues than in the spleen. Taken together, these data show that specific $CD8^+$ T memory cells rapidly turned over after a successful immunization with our vaccine and could again respond effectively to the next tumor cell challenge.

Cytotoxic T-cell response to a secondary D2F2 breast cancer cell challenge in severe combined immunodeficient mice. We could show that after vaccination with pUb-Fra-1/pIL-18 and subsequent tumor cell challenges, activated T cells can be successfully transferred adoptively to SCID mice where they also respond to a secondary challenge of D2F2 tumor cells. Specifically, SCID mice were challenged with D2F2 tumor cells after adoptive transfer of splenocytes from successfully immunized BALB/c mice. The data depicted in Fig. 7 indicate that putative $CD8^+$ T memory cells that had been parked for up to 30 days in SCID mice did effectively recognize a secondary challenge of D2F2 tumor cells. Furthermore, we observed that these activated $CD8^+$ T cells released increased amounts of IFN-γ (Fig. 7*A*) and were highly effective in cytotoxic killing of D2F2 breast cancer cells *in vitro* at different effector-to-target cell ratios (Fig. 7*B*).

Discussion

Fra-1, a transcription factor of the activator protein family, was shown previously to be involved in tumor cell progression and to be overexpressed in many human and murine tumor tissues (4–7). Here, we also provide evidence that Fra-1 is highly expressed in a variety of tumor cell lines at both the mRNA and protein levels. This finding suggests that Fra-1 could be linked to the malignancy of murine tumor cells and provides a potential target for immunotherapy of cancer, especially breast cancer cells. In fact, our previous work showed that a DNA vaccine targeting Fra-1 and coexpressing IL-18 could induce an effective cellular immune response, which led to the eradication of established D2F2 breast cancer metastases in syngeneic BALB/c mice (10). Here, we further extended our prior studies. Thus, we hypothesized that immunization with a DNA vaccine encoding murine Fra-1, fused to polyubiquitin and modified by cotransformation with a gene encoding secretory murine IL-18, will work effectively in two different breast carcinoma models as well as in a non–small cell lung carcinoma model and induce strong antitumor activity in syngeneic mice which can be maintained as a long-lived specific immune response against breast cancer cells. Meanwhile, IL-18 enhanced immune responses by activating T and NK cells while up-regulating MHC class I antigen expression and assisting the differentiation of $CD4^+$ T cells toward the Th1 subtype. Additionally, the effective generation and maturation of $CD8^+$ CTLs should result in an effective Th1 type immune response. Proof for this hypothesis was established by the induction of antitumor immune responses in three mouse tumor models, two of breast carcinoma (D2F2 and 4T1) and one non–small cell lung carcinoma (D121), both against primary tumors and their respective spontaneous and experimental pulmonary metastases. Our vaccine was also effective in a therapeutic setting of established pulmonary metastases. Three lines of evidence indicated that the effector cells responsible for this tumor protective immunity were primarily activated $CD8^+$ T cells. First, $CD8^+$ T cells isolated from splenocytes of specifically vaccinated mice specifically killed D2F2 and D121 target cells,

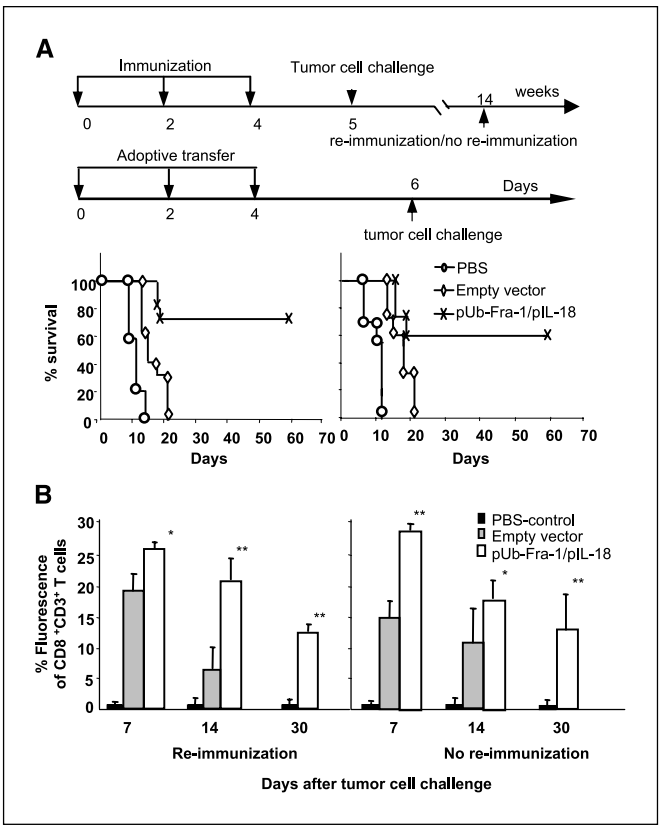


Figure 5. Maintenance of $CD8^+$ T memory cells in the absence of tumor antigen. *A,* vaccination schedule of donor mice was the same as that shown in Fig. 2 (i.e., three immunizations at 2-week intervals, followed by i.v. challenge with 0.5×10^6 D2F2 tumor cells 1 week after the last immunization). In the experimental group, 2 days after reimmunization at week 14 with pUb-Fra-1/pIL-18 or without reimmunization, lymphocytes were harvested from those mice that remained tumor free 2 months after the first tumor cell challenge. Lymphocytes (4×10^7/mouse) were adoptively transferred from BALB/c to SCID mice, and 2 days after transfer, these mice were challenged i.v. with 0.5×10^6 D2F2 tumor cells. Controls were mice adoptively transferred with lymphocytes harvested from control mice and immunized with only the empty vector or injected i.v. with PBS. Survival curves are representative of three separate experiments. *B,* fate of $CD8^+$ effector T cells after adoptive transfer into SCID mice was determined by two-color flow cytometric analysis of $CD8^+$ (PE labeled), $CD3^+$ (FITC labeled) splenic T cells, as well as by the effect of apoptosis on these cells after parking them in these mice 7 and 30 days, respectively. Differences between the two control groups (PBS and empty vector) and vaccine treatment groups were statistically significant. *, $P < 0.05$ and **, $P < 0.001$.

respectively in *in vitro* cytotoxicity assays. Second, the DNA vaccine did indeed activate $CD8^+$ T cells, because such cells isolated from splenocytes of successfully vaccinated mice, secreted the Th1 proinflammatory cytokine IFN-γ, and $CD8^+$ T-cell populations were markedly up-regulated. Third, $CD8^+$ T cells were activated both in lymphoid and nonlymphoid tissues, especially those located in tumor tissues of lung and liver. The mechanism of tumor protection is thought to depend on $CD4^+$ T cells and is potentially mediated by helper T cells, associated with effector functions and/or cytokine release which combine to break immunologic tolerance to tumor antigen. Our finding further supports this contention that both $CD4^+$ T cells and IL-2 release from these cells in the Peyer's Patches was markedly up regulated after one time vaccination.

The establishment and long-term maintenance of immunologic memory is a requirement for all protective vaccination strategies. Here, we showed that $CD8^+$ T cells isolated from splenocytes of successfully vaccinated BALB/c mice, when adoptively transferred to syngeneic SCID mice, maintained sufficient memory to markedly suppress dissemination and growth of a lethal challenge of D2F2 breast cancer cells. This finding was further supported by the markedly increased release of IFN-γ and $CD8^+$ T-cell cytotoxicity. Importantly, we found strong, local expression of $CD8^+$ T cells in tumor tissues, indicating that a $CD8^+$ T-cell response does not only occur in lymphoid tissues after successful vaccination, but that these T cells can also migrate to non-lymphoid tissues. This

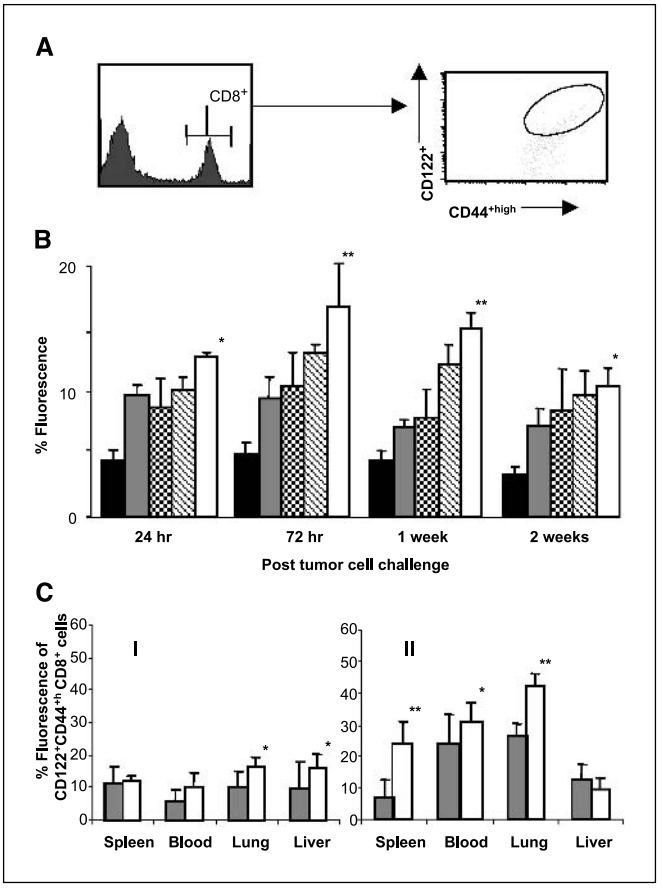


Figure 6. Turnover of memory $CD8^+$ T cells after vaccination with pUb-Fra-1/pIL-18. *A,* three-color flow cytometric analyses were performed of splenocytes obtained from immunized mice. Cells were stained with Cy-Chrome-labeled anti-$CD8^+$ antibody, PE-labeled anti-IL-2Rβ antibody, and FITC-labeled anti-$CD44^+$ antibody and analyzed and gated on live $CD8^+$ T cells. *B,* lymphocytes were isolated from groups of BALB/c mice treated with either pIL-18 (▨), pUb-Fra-1 (▩), and pUb-Fra-1/pIL-18 (□) or from groups of control mice treated only with PBS (■) or empty vector (▣) obtained at 24 and 72 hours, 1 and 2 weeks after tumor cell challenge and then analyzed by three-color flow cytometry, as described above. *Column,* mean for eight mice. Differences between the two control groups (PBS and empty vector) and the vaccine treatment groups were statistically significant *, $P < 0.05$ and **, $P < 0.001$. *C,* lymphocytes were isolated from spleen, lung, liver and blood of BALB/c mice of the treatment group pUb-Fra-1/IL-18 (□) 8 weeks after tumor cell challenge or from mice in the control group, that were only immunized with the (*I*) empty vector (■) or lymphocytes isolated from different tissues of SCID mice, adoptively transferred with either lymphocytes from mice immunized with pUb-Fra-1/pIL-18 (□) or with the empty vector (■) 1 week after tumor cell challenge (*II*). Three-color flow cytometric analyses were performed and cells stained with Cy-Chrome-labeled anti-$CD8^+$ antibody, PE-labeled anti-IL-2Rβ antibody, and FITC-labeled anti-CD44 antibody, gated on live $CD8^+$ T cells as described above. *Column,* mean for four mice. Differences between the control and treatment groups of mice were statistically significant when compared with lymphocytes from lungs, blood, and spleen of SCID mice after adoptive transfer following tumor cell challenge. This was also the case for lymphocytes from lungs and liver of syngeneic BALB/c mice obtained 8 weeks after tumor cell challenge. **, $P < 0.05$ and *, $P < 0.001$.

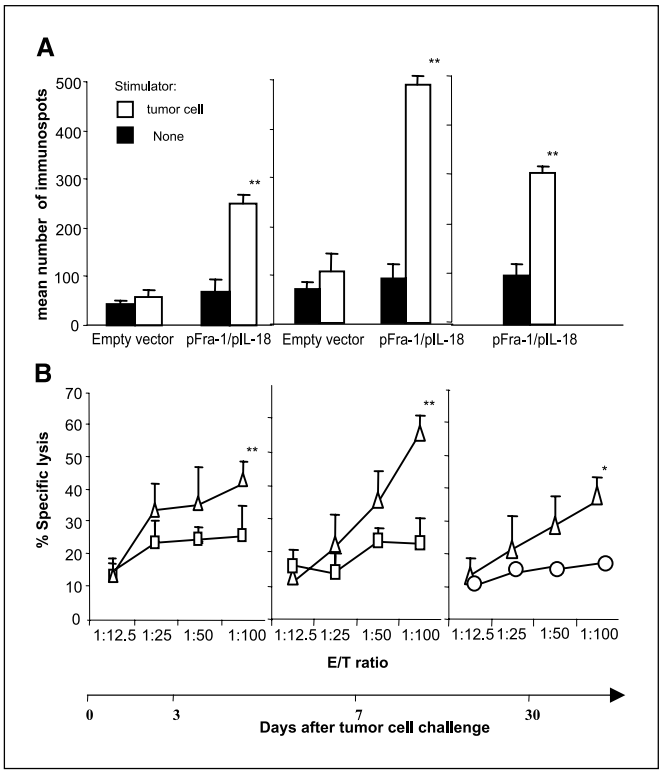


Figure 7. *A,* IFN-γ release from $CD8^+$ effector T cells, adoptively transferred to SCID mice as measured on days 2, 7, and 30 after i.v. challenge with D2F2 tumor cells. IFN-γ production of each experimental and control group of mice by ELISPOT assay is shown ($n = 8$, mean ± SD). Differences between the control group (empty vector) and the treatment group (pUb-Fra-1/pIL-18) are statistically significant **, $P < 0.001$. *B,* cytotoxicity induced by $CD8^+$ T cells, adoptively transferred to SCID mice. Splenocytes were isolated from mice, adoptively transferred with lymphocytes from successfully immunized BALB/c mice, and cytotoxity was measured on days 2, 7, and 30 after i.v. challenge with D2F2 tumor cells. Cytotoxicity was measured in a ^{51}Cr-release assay at different effector to target cell ratios. *Point,* mean of four mice. Differences between the two control groups of empty vector (□) and PBS (○) and the vaccine treatment group (△) were statistically significant *, $P < 0.05$ and **, $P < 0.001$.

occurs particularly in the tumor microenvironment where $CD8^+$ T cells react against antigen positive tumor cells. It is indeed relevant that some of these $CD8^+$ T cells were subsequently found as long-lived memory T cells that were ready to respond to the next stimulation upon re-encounter with the same tumor antigen. In this regard, it is well known that optimal T-cell activation results in clonal expansion, redistribution into nonlymphoid tissues and subsequent formation of memory (17, 18). It was also postulated that the immune response to foreign antigen is not necessarily limited to secondary lymphoid tissue. Importantly, nonlymphoid sites are essential for activated T-cell function and subsequent immunosurveillance. Most early studies of tumor antigen-specific T-cell responses were limited to analyses of lymph nodes, spleen, and blood. However, nonlymphoid tissues differ from organized secondary lymphoid organs in both, the quality and quantity of cytokines, lymphocytes, as well as immune accessory cells (19, 20). Importantly, we found that our DNA vaccine can induce protective antitumor immune responses, which not only occur in lymphoid tissues but also in nonlymphoid tissue near tumor sites and that, in addition, antigen-specific $CD8^+$ T cells can also migrate to nonlymphoid tissues and remain there for long periods of time as dormant memory cells. Strikingly, lymphocytes isolated from nonlymphoid tissues, such as lungs in our study, exhibited a greater release of IFN-γ and contained a higher percentage of $CD8^+$ CTLs as well as memory $CD8^+$ T cells than their splenic counterparts. These results point to the existence of a population of extralymphoid effector memory T cells poised for an immediate response to tumor-associated antigen. In fact, recent studies indicated that certain cytokines could induce bystander proliferation *in vivo* by T cells with a memory phenotype such as Type I IFN, IFN-γ, IL-15, IL-12, and IL-18. Moreover, it was found that injection of IL-18 stimulated a strong increase in the bromodeoxyuridine labeling of memory phenotype $CD8^+$ T cells *in vitro*. Furthermore, IFN-γ, which is inducible by IL-18, is also capable of promoting the turnover of memory phenotype $CD8^+$ T cells (21). Based on the finding that T-cell proliferation induced by IL-12 and IL-18 was dependent on IFN-γ (21), we examined the effect of IL-18 on T-cell turnover. Indeed, our results support the concept that coexpression of secretory IL-18 in our DNA vaccine induced the rapid turnover of $CD44^+$, $CD122^+$, $CD8^+$ T cells within 24 hours after immunization and that such $CD8^+$ T memory cells can be maintained in lymphoid tissues as well as locally in lung tumor tissues. Interestingly, $CD8^+$ T cells found in the lungs of our vaccinated mice were able to proliferate and acquire strong IFN-γ releasing capabilities after antigen exposure *in vitro*. Consequently, it is reasonable to conclude that persistently activated T cells and memory $CD8^+$ T cells in the lung can play a key role in the cellular immune response against tumor metastases.

The role of persisting antigen in T-cell memory cells and the requirement of such cells for chronic exposure to residual deposits of antigen for maintenance of $CD8^+$ T-cell memory have been the subject of much discussion and controversy (22–24). This is in contrast to $CD8^+$ effector T cells that absolutely do not require the presence of antigen. In fact, this is consistent with a decrease in adoptively transferred $CD8^+$ T cells observed in our studies in the absence of antigen (Fig. 5*A*). Indeed, a number of reports indicate that memory T cells survive poorly following adoptive transfer, unless accompanied by specific antigen (25–27). However, the contention that memory T cells require constant antigen stimulation has been challenged by reports demonstrating that $CD8^+$ memory cells can survive for prolonged periods of time after adoptive transfer in the absence of antigen (28–31). Our findings suggest that, at least for $CD8^+$ T cells, some memory T cells do not require continuous stimulation with antigen for survival.

In summary, we could show that an oral DNA vaccine, encoding Ub-Fra-1 and IL-18, carried by an attenuated strain of *S. typhimurium*, protected BALB/c mice against a lethal challenge of murine D2F2 and 4T1 breast cancer cells, and C57BL/6 mice against D121 lung carcinoma cell challenge. Moreover, this vaccine is capable of breaking T-cell tolerance to a self-antigen and generates a long-lived memory T-cell immune response against recurring breast cancer which could be maintained consistently in SCID mice in the absence of tumor antigen in both lymphoid and nonlymphoid organs.

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In re Patent Application of: Cheng Liu
Title: INHIBITING TUMOR CELL INVASION, METASTASIS AND ANGIOGENESIS
Docket No.: 1361.065PRV

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In re **PROVISIONAL** Patent Application of: Cheng Liu

Title: INHIBITING TUMOR CELL INVASION, METASTASIS AND ANGIOGENESIS

Docket No.: 1361.065PRV

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INVENTOR(s)/APPLICANT(s)

Name (last, first, middle initial)	RESIDENCE (CITY, AND EITHER STATE OR FOREIGN COUNTRY)
Liu, Cheng	**Carlsbad, CA**

TITLE OF THE INVENTION (280 characters max)

INHIBITING TUMOR CELL INVASION, METASTASIS AND ANGIOGENESIS

CORRESPONDENCE ADDRESS

Schwegman, Lundberg, Woessner & Kluth

Attn: Robin A. Chadwick

STATE	Minnesota	ZIP CODE	55402	COUNTRY	United States of America

ENCLOSED APPLICATION PARTS (check all that apply)

XXX	Specification	Number of Pages	122		Small Entity Status Claimed
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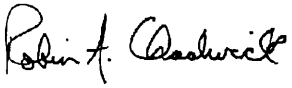

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INHIBITING TUMOR CELL INVASION, METASTASIS AND ANGIOGENESIS

This provisional application is related to PCT Application Ser. No. PCT/US2004/017157 filed May 28, 2004, which claims benefit of U.S. Application Ser. No. 60/474,840 filed May 29, 2003, both of which are incorporated by reference herein in their entirety.

Statement of Government Rights

The invention was made with the support of a grant from the Government of the United States of America (CDMRP Grant Numbers W81XWH-05-1-0091 and W81XWH-05-1-0318 from the Department of Defense). The Government may have certain rights to the invention.

Field of the Invention

The present invention relates to methods for treating and/or inhibiting tumor cell invasion, metastasis and/or angiogenesis by administering asparaginyl endopeptidase inhibitors. In some embodiments, the invention relates to prodrug agents useful for targeted delivery and activation of cytotoxic agents contained in the prodrug agents. The prodrugs become active within the tumor microenvironment of primary and metastatic tumor sites, as well as at the surface of cancerous cells and tumor stomal cells that express asparaginyl endopeptidases such as legumain.

Background of the Invention

The following description includes information that may be useful in understanding the present invention. It is not an admission that any of the information provided herein is prior art, or relevant, to the presently described inventions, or that any publication or document that is specifically or implicitly referenced is prior art.

According to the National Cancer Institute, since 1990 over 17 million people have been diagnosed with cancer, and an additional 1,334,100 new

cancer cases are expected to be diagnosed in 2003. About 556,500 Americans are expected to die of cancer in 2003, more than 1500 people every day. Cancer is therefore the second leading cause of death in the United States, exceeded only by heart disease. The National Institutes of Health estimate the overall costs of cancer in the year 2002 at $171.6 billion (Cancer Facts & Figures, 2003). Clearly, cancer is an enormous problem, and more effective cancer treatments are needed.

Two characteristic features of malignant cells are the ability to invade normal tissues and the ability to spread to distant sites. Tumor metastasis and invasion are the main cause of cancer mortality. Malignant cells can spread by several routes including direct local invasion, by the lymphatics or by capillaries. Local invasion is accomplished by an increase of tumor cell mobility and by production of proteases that destroy the normal extracellular matrix and basement membranes. Once the tumor cells escape from their normal boundaries, they are free to enter the circulation through the capillaries and the lymphatic system. The need for methods to prevent tumor invasion and metastasis is critical and constitutes a major goal in the effort to develop effective therapeutic interventions against cancer.

In addition, many cancer cells are capable of inducing angiogenesis. To form blood vessels, angiogenic endothelial cells share some of the same biochemical mechanisms that are used by cancer cells to invade local tissues.

Current cancer treatments generally involve the use of surgery, radiation therapy, and/or chemotherapy. However, these treatments all involve serious side effects. For example, surgery can be complicated by bleeding, damage to internal organs, adverse reactions to anesthesia or other medicines, pain, infection, and slow recovery. Radiation therapy can damage normal cells and can cause fatigue. For many people, chemotherapy is the best option for controlling their cancer. However, chemotherapy can also damage normal cells such as bone marrow and blood cells, cells of the hair follicles, and cells of the reproductive and digestive tracts. Chemotherapy can also cause nausea, vomiting, constipation, diarrhea, fatigue, changes to the nervous system, cognitive changes, lung damage, reproductive and sexual problems, liver, kidney, and urinary system damage, and, especially with the use of the

chemotherapeutic agent doxorubicin, heart damage. Long-term side effects of chemotherapy can include permanent organ damage, delayed development in children, nerve damage, and blood in the urine. Thus, the use of the chemotherapy for cancer treatment is not without serious side effects.

Most agents currently administered to a patient are not targeted to the site where they are needed, resulting in systemic delivery of the agent to cells and tissues of the body where the agent is unnecessary, and often undesirable. Such systemic delivery may result in adverse side effects, and often limits the dose of an agent (*e.g.*, cytotoxic agents and other anti-cancer agents) that can be administered. Accordingly, a need exists for agents and methods that specifically target cancerous cells and tissues.

Thus, it would be desirable to be able to direct various agents to cancer cells and to the tumor microenvironment so as to be able to decrease the dosage of the agents given and to decrease the systemic toxicity and side effects associated with these agents.

Summary of the Invention

According to the invention, an unexpectedly high level expression of asparaginyl endopeptidases, including legumain, is present in a wide variety of cancer cells, particularly those involved in metastasis. As described herein, legumain expression occurs early in the development of cancer cell invasion, just as metastasis begins, and under the hypoxic conditions associated with invasive tumor growth. Also as shown herein, legumain activity is substantially increased on the surface of tumor cells by cell-surface association with integrins, indicating that integrins are co-factors for legumain. Moreover, legumain can activate metalloproteinases (e.g. MMP-2) and cathepsins (e.g., cathepsins B, H and L), which are all proteases involved in promting tumor cell invasion and metastasis. In addition, asparaginyl endopeptidase expression is also associated with reduced cancer cell apoptosis and increased angiogenesis. Therefore, asparaginyl endopeptidase expression and activity is a cancer and angiogenesis marker and an indicator of tumor cell metastasis. The invention therefore provides agents to treat undesirable angiogenesis, tumor cell invasion, tumor cell metastasis and other such cancerous conditions, particularly those conditions

involving cells and tissues that express asparaginyl endopeptidases, including legumain.

Many tumor cells are largely resistant to chemotherapy, for example, because the chemotherapeutic agents employed are only active against a subset of the tumor cells that comprise a cancerous condition. According to the invention, stromal cells in the tumor microenvironment, such as endothelial cells or tumor associated macrophages (TAMs), can be used to effectively treat these drug-resistant cancer cells. This strategy is also effective for reducing the expression and/or activity of molecules in the tumor microenvironment that attract TAMs and other tumor-associated cells that facilitate tumor growth and invasion. TAMs consist of a polarized M2 (CD206+, F4/80+) macrophage population with little cytotoxicity for tumor cells because of their poor production of nitric oxide and proinflammatory cytokines. TAMs also possess poor antigen presenting capacity and effectively suppress T cell activation. In fact, TAMs actually promote tumor cell proliferation and metastases by producing a wide range of growth factors, pro-angiogenesis factors, metalloproteinases and the like. TAMs also partake in circuits that regulate the function of fibroblasts in the tumor stroma and are particularly abundantly expressed in the tumor stroma.

According to the invention, TAMs express high levels of legumain in the tumor microenvironment. In contrast, classical macrophages of the M1 phenotype, that perform key immune-surveillance functions, do not express legumain. Consequently, targeted elimination of TAMs does not interfere with the biological functions of normal (MI) macrophages, including cytotoxicity and antigen presentation. Thus, one aspect of the invention involves targeting legumain-expressing TAMs with prodrugs and/or legumain inhibitors to destroy TAMs and/or inhibit their function.

TAM and endothelial cells are non-transformed cells therefore will not develop drug resistance that is common among malignant cancers. Thus, low dosages of the prodrugs and/or legumain (asparaginyl endopeptidase) inhibitors can be employed when targeting these TAM and endothelial cells. This will down regulate a wide variety of tumor growth factors, pro-angiogenesis factors

and enzymes released by these macrophages and lead to inhibition of tumor angiogenesis as well as invasive growth and metastasis.

One aspect of the invention is a method of reducing the growth or function of tumor stromal cells in a mammal that includes administering to the mammal an effective amount of a prodrug or an asparaginyl endopeptidase inhibitor. Examples of stromal cells that can be treated by the methods of the invention include tumor-associated macrophages and endothelial cells.

Another aspect of the invention is a method of inhibiting angiogenesis that involves contacting a cell capable of angiogenesis with an asparaginyl endopeptidase inhibitor. A related aspect of the invention is a method of inhibiting angiogenesis that involves contacting a cell capable of angiogenesis with a prodrug of the invention.

Examples of asparaginyl endopeptidase inhibitors (AEPIs) that may be used in the methods of the invention have the following structures:

AEPI-1 with Cbz-Ala-Ala-AzaAsn-(S,S)-EPCOOEt;

AEPI-2 with Cbz-Ala-Ala-AzaAsn-CH=CH-COOEt;

AEPI-3 with Cbz-Ala-Ala-AzaAsn-CH=CH-COOBzl; and

AEPI-4 with Cbz-Ala-Ala-AzaAsn-CH=CH-CON(CH_3)Bzl, where Cbz is benzyloxycarbonyl, Et is ethyl and Bzl is benzyl.

Another aspect of the invention is a legumain-activated prodrug that is tumoricidal *in vivo*, with reduced side effects and toxicity relative to currently available chemotherapeutics. These agents are useful not only to treat cancer, but also useful to treat other conditions and cells that involve legumain expression, including non-trasformed cells that support tumor growth and invasion. The prodrug compound includes a drug molecule linked to a peptide, wherein the peptide has an amino acid sequence that includes at least two linked amino acids, wherein at least one of the two linked amino acids is Asn, and wherein legumain cleaves the peptide at the covalent linkage between the Asn and another amino acid to generate an active drug from the prodrug. The prodrug is substantially non-toxic to normal animal cells, whereas the drug is an active drug that can have a beneficial effect upon an animal to which it is administered. Such a compound can be, for example, N-(succinyl-L-Ala-L-Ala-L-Asn-L-Leu)doxorubicin and N-(succinyl-L-Ala-L-Ala-L-Asn)taxel.

The drug employed is any drug whose action is diminished or blocked by attachment of a peptide to the drug. The ability of the drug to enter cells is diminished, inhibited or blocked by attachment of the peptide and hydrophilic groups. Such hydrophilic groups are generally included to facilitate water-solubility and cell impermeability. Hydrophilic groups are generally attached to the peptide so that the function of the drug is not inhibited or blocked by the hydrophilic group once the peptide is cleaved from the prodrug to yield the drug.

In some embodiments, the drug can be a cytotoxin. Such a cytotoxin can be aldesleukin, bleomycin sulfate, camptothecin, carboplatin, carmustine, cisplatin, cladribine, lyophilized cyclophosphamide, non-lyophilized cyclophosphamide, cytarabine, dacarbazine, dactinomycin, daunorubicin, diethyistilbestrol, epoetin alfa, esperamycin, etidronate, etoposide, filgrastim, floxuridine, fludarabine phosphate, fluorouracil, goserelin, granisetron hydrochloride, idarubicin, ifosfamide, immune globulin, interferon alpha-2a, interferon alpha-2b, leucovorin calcium, leuprolide, levamisole, mechiorethamine, medroxyprogesterone, melphalan, methotrexate, mitomycin, mitoxantrone, octreotide, ondansetron hydrochloride, paclitaxel, pamidronate, disodium, pegaspargase, plicamycin, sargramostim, streptozocin, taxol, thiotepa, teniposide, vinblastine, or vincristine. In some embodiments, the drug is doxorubicin or paclitaxel..

In one embodiment, the prodrug can have a peptide amino acid sequence comprising SEQ ID NO:3:

$$\text{Hyd-(Xaa1)}_n\text{-Xaa2-Asn-(Xaa3)-drug}$$

wherein:

Hyd is a hydrophilic group;

n is an integer of about 0 to about 50;

Xaa1 and Xaa2 are separately any amino acid;

Xaa3 is either nothing or an amino acid that has no substantial effect on the activity of the drug; and

the drug employed is a drug whose action is diminished or blocked by attachment of a peptide to the drug.

Examples of peptide sequences that may be used include amino acid sequence Asn-Leu, Ala-Asn-Leu, Thr-Asn-Leu, Ala-Ala-Asn-Leu (SEQ ID

NO:5), Ala-Thr-Asn-Leu (SEQ ID NO:6), and Boc-Ala-Ala-Asn-Leu (SEQ ID NO:4). Examples of prodrugs provided by the invention include Boc-Ala-Ala-Asn-Leu-doxorubicin (SEQ ID NO:7), succinyl-Ala-Ala-Asn-Leu-doxorubicin (SEQ ID NO:8), N-(-*t*-Butoxycarbonyl-Ala-Thr-Asn-Leu)doxorubicin (SEQ ID NO:9), N-(Succinyl-Ala-Thr-Asn-Leu)doxorubicin (SEQ ID NO:10), N-(-*t*-Butoxycarbonyl-Ala-Asn-Leu)doxorubicin (SEQ ID NO:11), N-(Succinyl-Ala-Asn-Leu)doxorubicin (SEQ ID NO:12), N-(-*t*-Butoxycarbonyl-Thr-Leu)doxorubicin (SEQ ID NO:13), N-(Succinyl-Thr-Leu)doxorubicin (SEQ ID NO:14),

As described above the Hyd group facilitates prodrug and inhibitor water solubility and inhibits cell uptake and tissue retention of the prodrug before activation and of the inhibitor before binding to a legumain:integrin complex. In some embodiments, Hyd is a succinyl group. In other embodiments, Hyd is a hydrophilic protecting group. A variety of hydrophilic protecting groups can be utilized, for example, in some embodiments the protecting group is a hydrophilic amino protecting group. In other embodiments, the protecting group is succinyl. Less hydrophilic groups that can used in place of succinyl include *t*-butoxycarbonyl or *N*-β-alanyl.

Anothet aspect of the invention is a pharmaceutical composition that includes at least one of the prodrug compounds of the invention and a pharmaceutically acceptable carrier. In some embodiments, the carrier is a liposome. Combinations of the present prodrug compounds can also be included in the compositions of the invention.

The invention also provides a method for treating a mammal having, or suspected of having cancer. The method includes administering to the mammal a prodrug compound of the invention in amounts and at intervals effective to prevent, reduce, or eliminate one or more of the symptoms associated with cancer. The cancer can be an invasive or metastatic cancer. The cancer can also be a tumor that is prone to angiogenesis. Cancers that can be treated by the invention include solid tumors and cancers as well as cancers associated with particular tissues, including breast cancer, colon cancer, lung cancer, prostate cancer, ovarian cancer, cancer of the central nervous system, carcinomas, lymphomas, melanomas, fibrosarcomas, neuroblastoma, and the like. The

cancer can, for example, be autoimmune deficiency syndrome-associated Kaposi's sarcoma, cancer of the adrenal cortex, cancer of the cervix, cancer of the endometrium, cancer of the esophagus, cancer of the head and neck, cancer of the liver, cancer of the pancreas, cancer of the prostate, cancer of the thymus, carcinoid tumors, chronic lymphocytic leukemia, Ewing's sarcoma, gestational trophoblastic tumors, hepatoblastoma, multiple myeloma, non-small cell lung cancer, retinoblastoma, or tumors in the ovaries.

The invention also provides a method for imaging a tissue, that includes contacting a test tissue suspected of including legumain with of an agent that specifically binds to legumain, and detecting whether the agent binds to the test tissue. The method can further include quantifying and comparing amounts of the agent bound to the test tissue with amounts of the agent bound to a control tissue that does not comprise legumain. The agent can be an antibody that specifically binds to legumain.

The invention also provides a method for diagnosing cancer in a tissue that includes contacting the tissue with of an agent that specifically binds to legumain, and detecting whether the agent binds to the tissue. The invention also provides a method for diagnosing cancer in an animal that includes administering to the animal an agent that specifically binds to legumain, and detecting whether the agent accumulates in a tissue. These methods can further include diagnosing the patient as having or not having cancer.

The invention also provides a method for inhibiting cancer metastasis and/or tumor cell invasion in an animal, including administering a compound or prodrug of the invention to the animal in amounts and at intervals effective to prevent, reduce, or eliminate cancer metastasis and/or tumor cell invasion.

The invention also provides a method for inhibiting cell migration in an animal that includes administering a compound or prodrug of the invention to the animal in amounts and at intervals effective to prevent, reduce, or eliminate cancer cell migration.

The invention also provides a method of killing a cell in a tissue, including contacting the cell with a compound or prodrug of the invention in amounts and at intervals effective to kill the cell, wherein the tissue includes cells that express legumain.

The invention also provides a method for treating cancer in animal that includes administering to the animal a compound of the invention that inhibits legumain in amounts and at intervals effective to prevent, reduce, or eliminate one or more symptoms of cancer in the animal.

The invention also provides a method for inhibiting cancer metastasis in a tissue that includes contacting the tissue with a compound that inhibits legumain in amounts and at intervals effective to prevent, reduce, or eliminate cancer metastasis.

The invention also provides a method for inhibiting cancer cell migration in a tissue that includes contacting the tissue with a compound that inhibits legumain in amounts and at intervals effective to prevent, reduce, or eliminate cancer cell migration.

The invention also provides a method for treating inflammation in an animal, which includes administering to the mammal a compound that inhibits legumain in amounts and at intervals effective to prevent, reduce, or eliminate one or more symptoms associated with cancer.

The invention also provides a method for delivering a drug to a legumain-expressing cell in a mammal, which includes administering to the mammal an effective amount of a drug attached an agent that binds to legumain. The agent that binds to legumain can be a legumain inhibitor, a legumain substrate, an anti-legumain antibody or other agent that can bind to legumain.

The invention also provides a legumain inhibitor having including formula **III**, **IV** or **V**:

$$\text{Hyd-(Xaa4)}_n\text{-Asn-CHO} \qquad \textbf{III}$$

$$\text{Xaa4-Asn-Y} \qquad \textbf{IV}$$

$$\text{Xaa4-azaAsn-Y} \qquad \textbf{V}$$

wherein:

Hyd is a hydrophilic group that may also serve as a protecting group;

n is an integer of about 2 to about 5;

Xaa4 is an amino acid or an amino acid mimetic;

Y is alkyl, alkenyl epoxide or Michael acceptor, optionally substituted with 1-3 halo or hydroxy, alkylamino, dialkylamino, alkyldialkylamino, or cycloalkyl, alkylcycloalkyl, alkenylcycloalkyl, aryl; (C_5 -C_{12})arylalkyl or (C_5 -C_{12})arylalkenyl,

wherein the aryl groups of the arylalky or arylalkenyl can be 0-4 heteroatoms selected from N, O and S, and are optionally substituted with halo, cyano, nitro, haloalkyl, amino, aminoalkyl, dialkylamino, alkyl, alkenyl, alkynyl, alkoxy, haloalkoxy, carboxyl, carboalkoxy, alkylcarboxamide, (C_5 -C_6)aryl, --O-(C_5 -C_6)aryl, arylcarboxamide, alkylthio or haloalkylthio; and

wherein the inhibitor is capable of binding to legumain.

In other embodiments, the legumain inhibitor can be cystatin, stefin, a peptide including the sequence Ala-Leu-β-Asn-Ala-Ala (SEQ ID NO:15) or an antibody that inhibits legumain activity.

In another embodiment, the compounds of the invention can be used for the manufacture of a medicament useful for treating diseases such as cancer.

Brief Description of the Figures

This patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

FIG. 1A-F illustrates that legumain is over-expressed in tumors. FIG. 1A provides doubly-stained section of CT26 mouse colon cancer. Legumain stained red and CD31+ endothelial cells stained green in the original (magnification 600×). Legumain expression was high in tumor cells. Endothelial cells also expressed legumain. Legumain appeared to be largely in membranous vesicles, consistent with a distribution of endosomes/lysosomes. Legumain was also detected on the surface of tumor cells and endothelial cells (arrows). FIG. 1B provides a western blot analysis of legumain expression. Lanes 1- 9 are brain, tumor, lung, heart, muscle, intestine, spleen, liver, and kidney, respectively. Legumain expression is high in tumor tissues (lane 2). Legumain expression in normal tissues is highest in kidney (lane 9), followed by liver (lane 8) and spleen

(lane 7). FIG. 1C illustrates whether legumain expression can be detected with anti-legumain antisera in the following normal human tissues: kidney, adrenal gland, bone marrow, lymph, muscle, ovary, colon, lung and prostate (magnifications 200×). FIG. 1D illustrates whether legumain expression can be detected with anti-legumain antisera in the following normal human tissues: cerebellum, liver, heart, esophagus, pancreas, peripheral nerve, stomach, testis and thyroid (magnifications 200×). FIG. 1E illustrates whether legumain expression can be detected with anti-legumain antisera in the following tumor specimens: breast cancer, CNS cancer, lymphoma, and melanoma (magnification 400×). FIG. 1F illustrates whether legumain expression can be detected with anti-legumain antisera in the following tumor specimens: colon cancer, lung cancer, ovarian cancer, and prostate cancer (magnification 400×).

FIG. 2A-G illustrates the cellular distribution and activity of legumain. FIG. 2A shows that legumain is detected in intracellular vesicles (lighter areas; green staining in the original). FIG. 2B shows that legumain is prominently associated with the invadopodia of migrating tumor cells (arrows). FIG. 2C shows that legumain is also observed on cell surface of serum starved BEND3 cells (arrows). FIG. 2D shows that legumain is associated with the actin cortex (arrows). FIG. 2E show doubly-stained legumain^{+} 293 cells where legumain stained in red and integrin β1 stained in green in the original. Legumain appeared in granular organelles that resemble aggregated lysosomes, as well as on the cell surface, co-localizing with β1 integrins (arrow). Magnification 1,000×. FIG. 2F illustrates the conversion of a 72 kDa progelatinase A to the 62 kDa active enzyme by legumain. Activation was minimal in reaction with control 293 cells (lane 1), but a majority of this zymogen was converted to active when reacted with legumain^{+} 293 cells (lane 2). Activation was fully inhibited by cystatin (lane 3). FIG. 2G illustrates that legumain was not active against progelatinase B (lane 4 and lane 5 are progelatinase B with 293 cells and legumain+ 293 cells, respectively).

FIG. 3A-B shows that legumain expression promotes cell migration and invasion. FIG. 3A illustrates that the migration of legumain+ 293 cells was markedly greater than the migration of control 293 cells. The enhanced cell migration was partially inhibited by cystatin, but not by TIMP-2 or E64. FIG.

3B shows that legumain enhanced 293 cell invasion across a matrigel barrier as compared to control 293 cells that did not overexpress legumain. The invasive activity was partially inhibited by cystatin and TIMP-2, but was not affected by the presence of E64. Each bar represented the mean ±SE of three independent wells, and the experiments were repeated at least three times with similar results. $p < 0.001$.

FIG. 4A-C shows that legumain enhances tumor invasion and metastasis *in vivo*. FIG. 4A illustrates that distant metastases were detected in 50% of WEHI nude mice inoculated with 293 cells that over-expressed legumain as compared to mice inoculated with wild type 293 cells. FIG. 4B provides a photomicrograph of a section of a tumor generated in WEHI nude mice with legumain$^+$ 293 cells. FIG. 4C provides a photomicrograph of a section of a tumor generated by control 293 cells. Note that the pseudo-encapsulation seen in the typical control 293 cell tumors (arrows) was lacking in legumain$^+$ 293 cell tumors. Moreover, 293 tumor invasion of muscle was frequent in mice receiving legumain$^+$ 293 cells (arrows, FIG. 4B). Magnification 200×.

FIG. 5A provides a chemical structure for the legubicin prodrug that can be activated by the asparaginyl endopeptidase legubicin, where an amino group of the doxorubicin is covalently bonded to the carboxy terminus of a leucine residue. FIG. 5B graphically illustrates the cytotoxicity of legubicin and doxorubicin in legumain$^+$ 293 cells and in control 293 cells. The following symbols were employed in the graph: ♦\solid diamond) 293 cells treated with legubicin; ■ (solid square) 293 cells treated with doxorubicin; ▲ (solid triangle) legumain expressing 293 cells treated with legubicin; and ▼ (upside down solid triangle) legumain expressing 293 cells treated with doxorubicin. Cytotoxic activity of legubicin is much higher on legumain$^+$ cells than on control 293 cells, consistent with legumain activation of the prodrug by these cells.

FIGs. 6A-E illustrate the tumoricidal effect of legubicin on CT26 colon carcinoma *in vivo*. FIGs. 6A1-6A3 graphically illustrate the *in vivo* effect of legubicin on CT26 colon carcinoma tumor volume (FIG. 6A1 and 6A2) and animal weight (FIG. 6A3). Three intraperitoneal injections at both 5 mg/kg and 50 mg/kg were administered with 2 day intervals. Legubicin arrested tumor growth and tumor eradication was achieved (FIG. 6A1 and 6A2) with little

evidence of toxicity, as indicated by animal weight loss (FIG. 6A3). In contrast, doxorubicin caused the death of the host animals at 5 mg/kg. FIG. 6A2 is a graph of the same experiment presented in FIG. 6A1 without data from the mock treated control group. FIG. 6B provides a photomicrograph of a H&E stained tumor section, where the animal had been treated with legubicin (magnification 1320×). FIG. 6C provides a photomicrograph of a H&E stained tumor section, where the animal had been treated with equivalent dose of doxorubicin (magnification 1320×). FIG. 6D provides a photomicrograph of a tumor section that had been subjected to a TUNEL assay, where the tumor specimens were treated with legubicin (magnification 400×). FIG. 6E provides a photomicrograph of a tumor section that had been subjected to a TUNEL assay, where the tumor specimens were treated with doxorubicin (magnification 400×). As shown in FIGs. 6D and 6E, tumors treated with legubicin have a higher apoptotic index than tumors treated with doxorubicin (see arrows).

FIG. 7A-C shows that legumain expression is induced by hypoxia and occurs early in metastatic sites. FIG. 7A illustrates a Western blot analysis of legumain expression in cells under normoxic and hypoxic conditions. FIG. 7B shows a hematoxylin and eosin stained section of 4T1 breast carcinoma lung metastasis. FIG. 7C provides results of an immunohistochemical analysis of frozen section of 4T1 lung metastasis. Legumain is stained green, CD31 is stained red, and nuclei are stained blue. The metastatic site expressing legumain is indicated by white arrows. Yellow arrows indicate auto-fluorescence of wall of a small bronchiole.

FIG. 8A-E shows that legumain forms a legumain:αvβ3 integrin protease complex. FIG. 8A shows that legumain is immunoprecipitated with a panel of anti-integrin antibodies. The anti-integrin antibody type is indicated above each lane of the western blot. FIG. 8B shows that legumain is co-precipitated from cell lysates using anti-αvβ3 integrin antibodies and αvβ3 integrin is co-precipitated from cell lysates with anti-legumain antibodies. FIG. 8C illustrates the location of legumain:αvβ3 integrin complexes in MBA-MA231 cells. The αvβ3 integrin is red, legumain is green, nuclei are blue and the legumain:αvβ3 complexes are yellow. These legumain:αvβ3 complexes are formed intracellularly in vesicles and transported to cell surfaces. FIG. 8D illustrates

extensive formation of legumain:αvβ3 integrin complexes in Panc-1 human pancreatic carcinoma grown in nude mice. The αvβ3 is red, legumain is green, cell nuclei are blue and the legumain:αvβ3 co-localization is yellow. FIG. 8E provides a schematic representation of legumain:αvβ3 complexes and the types of intracellular trafficking and cell surface proteolysis that occur.

FIG. 9A-E shows that integrin αvβ3 is co-factor of legumain activity. FIG. 9A shows that binding of αvβ3 integrin increases the amidolytic activity of legumain in a dose dependent manner. FIG. 9B provides a comparison of asparaginyl endopeptidases activity of legumain (dark bars) and legumain: αvβ3 complexes (open bars). FIG. 9C shows that αvβ3 integrin shifts the pH dependency of legumain to a higher pH. FIG. 9D shows that αvβ3 integrin increases legumain activity towards its physiologic substrate MMP2 as assessed using zymogram. FIG. 9E provides a schematic illustration of MMP2 activation by legumain. Legumain not only activates MMP2 by removing its propeptide, it also cleaves MMP2 between the MMP2 catalytic domain and the hemopexin-like repeats. Hemopexin can inhibit angiogenesis, indicating that legumain has a regulatory role in vessel development.

FIG. 10A-G shows that legumain is an important regulator of hypoxia induced endothelial invasion and tube formation. FIG. 10A shows the structures of four asparaginyl endopeptidases inhibitors (AEPIs) that legumain inhibitory activity. The four AEPI's have the following chemical names: AEPI-1 is Cbz-Ala-Ala-AzaAsn-(S,S)-EPCOOEt; AEPI-2 is Cbz-Ala-Ala-AzaAsn-CH=CH-COOEt; AEPI-3 is Cbz-Ala-Ala-AzaAsn-CH=CH-COOBzl; and AEPI-4 is Cbz-Ala-Ala-AzaAsn-CH=CH-CON(CH3)Bzl. FIG. 10B illustrates the cytotoxicity of AEPIs in wild type 293 cells and in 293 cells that express legumain. FIG. 10C shows that the four AEPIs have strong inhibitory activity as assessed by a legumain amidolytic assay. FIG. 10D shows that increasing concentrations of AEPI-1 increasingly inhibit HUVEC tube formation in vitro. FIG. 10E shows that addition of recombinant legumain protein promotes HUVEC tube formation. FIG. 10F illustrates HUVEC matrigel tube formation under normal and hypoxic conditions, with and without legumain inhibitors cystatin and aza-peptide epoxide. FIG. 10G illustrates the number of migrating cells per field as detected

in invasion assays of HUVEC under normal and hypoxic conditions, with and without legumain inhibitors, cystatin and aza-peptide epoxide.

FIG. 11A-D shows that AEPI-1 inhibits angiogenesis *in vivo*. FIG. 11A shows that AEPI-1 inhibits mouse aorta vessel sprouting in a dose-dependent manner. FIG. 11B further illustrates that AEPI-1 inhibits the extension of mouse aorta vessel sprouts after the sprouts had already formed. FIG. 11C shows that AEPI-1 inhibits angiogenesis within FGF-2 and cancer cell containing Matrigel plugs that were injected subcutaneously into mice. Vessel density was quantified by hemoglobin content in the plugs. FIG. 11D provides representative histology sections of angiogenic vessels induced by FGF-2 in the Matrigel plugs (top panels), and CD31 positive vasculatures in Matrigel plugs that were induced by cancer cells (bottom panels).

FIG. 12A-D shows that AEPI-1 suppresses tumor invasive growth. FIG. 12A shows that legumain promotes tumor cell invasion in vitro. FIG. 12B illustrates the human breast cancer model established in nude mouse. The tumor is similar to a human infiltrating ductal carcinoma with apparent glandular differentiation. The tumors grow aggressively and are invading surrounding local muscles. FIG. 12C shows that AEPI-1 treatment of tumors suppresses cancer cell differentiation and invasive growth. FIG. 12D shows that AEPI-1 treatment of tumors dramatically reduces tumor volume as assessed at 14 days post cancer cell injection.

FIG. 13A-C illustrates legumain expression in cancer cells and cell surface association of legumain. FIG. 13A provides results of a Western blot analysis of cultured tumor cells and corresponding *in vivo* tumor derived cells. FIG. 13B shows a flow cytometry analysis of cell surface legumain in single cell suspensions obtained from CT26 tumors with (bottom panel) and without (top panel) collagenase treatment. FIG. 13C shows flow cytometry analysis of single cell suspensions prepared from tumor, bone marrow, spleen, and kidney tissues as well as cultured CT26 tumor cells for cell surface legumain. The percentage of cells positive for surface legumain is plotted in the bar graph insert.

FIG. 14A-E shows that legumain is expressed by stromal cells in tumors. FIG. 14A illustrates that double staining with anti-legumain antibody (green) and anti-CD31 antibody (red) identifies endothelial cells. Tumor vascular

endothelial cells expressing legumain in tumor are indicated by arrows (400x). FIG. 14B illustrates that double staining of anti-legumain antibody (green) and anti-CD68 antibody (red) identifies legumain expressing TAMs (arrows) (600x). TAM cells expressing legumain are indicated by arrows. FIG. 14C illustrates that double staining of anti-legumain (green) with anti-collagen I antibody (red) (400x). Co-localization of legumain with collagen I in the extracellular matrix is indicated by yellow. FIG. 14D provides a two dimensional analysis of single cell suspensions prepared from 4T1 *in vivo* mouse mammary tumors following mechanical dissociation and of 4T1 tumor cells from tissue culture. Legumain was observed on the surface of viable endothelial cells and tumor-associated macrophages as identified by anti-legumain antibodies and anti-CD31 antibodies or anti-CD14 antibodies, respectively. FIG. 14E illustrates two dimensional flow cytometry of single cell suspensions prepared from 4T1 tumors using collagenase digestion. Legumain-associated endothelial cells constituted the group of cells that are legumain$^+$ and CD31$^+$. Legumain-associated TAMs constituted legumain$^+$ and CD14$^+$ cells.

FIG. 15A-E illustrates the structures and activities of LEG family compounds. FIG. 15A shows the structures of three legumain prodrugs (LEG-2, LEG-3 and LEG-4). LEG-2 (*N*-Succinyl-β-alanyl-L-threoinyl-L-Asparaginyl-L-Leucyl-Doxorubicin) and LEG-3 (*N*-Succinyl-β-alanyl-L-alanyl-L-Asparaginyl-L-Leucyl-Doxorubicin) are oligopeptidic derivatives of doxorubicin that are cell impermeable and can be hydrolyzed to leucine-doxorubicin by extracellular legumain. LEG-4 (*N*-Succinyl-β-alanyl-L-Asparaginyl-L-Leucyl-Doxorubicin) is similar but is not subject to legumain hydrolysis. FIG. 15B illustrates the cytotoxicity of LEG compounds using legumain$^+$ 293 cells and control wild-type 293 cells. FIG. 15C illustrates legumain-mediated LEG activation and inhibition by cystatin. Bar (1) Untreated control; bar (2) Dox treated; bar (3) Dox plus cystatin; bar (4) LEG-3 treated; bar (5) LEG-3 plus cystatin; bar (6) LEG-2 treated; and bar (7) LEG-2 plus cystatin. FIG. 15D illustrates cellular uptake of LEG-3 compared to Dox. FIG. 15E shows localization of Dox in cell nuclei is visualized by auto-fluorescence (red). In contrast, LEG-3 is not internalized by legumain negative cells exposed to LEG-3 and they lack nuclear positivity for end product Dox despite presence of extracellular fluorescent signal of the Dox

present in the LEG-3.

FIG. 16A-C illustrates the *in vivo* distribution and pharmacokinetics of LEG-3. FIG. 16A shows the plasma pharmacokinetics of LEG-3 compared to Dox. When Dox was administered, the plasma concentration very rapidly declined and then a low Dox concentration was detected that slowly cleared. In contrast, the plasma concentration of LEG-3 remained higher than that of Dox for a longer period of time. FIG. 16B shows accumulation of LEG-3 (dark bars) and Dox (light bars) in organs and tumors of mice bearing CT26 colon carcinomas. FIG. 16C illustrates the presence of Dox and LEG-3 in cells as visualized with auto-fluorescence of Dox (red).

FIG. 17A-B shows that LEG-3 lacks the *in vivo* toxicity of Dox. FIG. 17A illustrates the myelosuppression of LEG-3 and Dox in mice as assessed by determination of the numbers of peripheral blood leukocytes. FIG. 17B illustrates the toxicity of LEG-3 in cardiac tissues as demonstrated by the presence of vacuolar degeneration of myocytes in hematoxylin and eosin stained cardiac sections. Chronic Dox treatment resulted in large numbers of vacuoles. In contrast, vacuoles were notably absent in the cells of LEG-3 treated mice. TUNEL analysis of cardiac tissue from mice treated with Dox also demonstrated marked apoptosis of myocytes (red apoptotic nuclei are indicated by arrows). This was very infrequent in LEG-3 treated mice.

FIG. 18A-F illustrates the *in vivo* specificity and efficacy of LEG-3 in syngeneic mouse tumor models. FIG. 18A illustrates the *in vivo* effects of LEG-4 in mice bearing CT26 tumors (n=8). FIG. 18B illustrates the *in vivo* effects of LEG-2 in mice bearing CT26 tumors (n=8), by showing hematoxylin and eosin stained sections of control versus treated tumors. FIG. 18C illustrates the *in vivo* effects of LEG-3 (n=8), by showing hematoxylin and eosin stained sections of control versus treated tumors. FIG. 18D shows the *in vivo* effects of LEG-3 in A/J mice bearing C1300 neuroblastomas (n=8), by showing hematoxylin and eosin stained sections of control versus treated tumors. FIG. 18E graphically illustrates results of a survival analysis of CT26 tumor bearing mice treated with LEG-3. FIG. 18F graphically illustrates results of a survival analysis of C1300 bearing mice treated with LEG-3.

FIG. 19A illustrates the efficacy of LEG-3 in human xenograft tumor

models compared to Dox. FIG. 19A graphically illustrates the *in vivo* effects of LEG-3 (49.4 μmol/kg) (n=8) compared to Dox (1.72 μmol/kg) (n=8) in the HT1080 human fibrosarcoma model and hematoxylin and eosin stained histological analysis of control HT1080 tumor compared with tumors treated with Dox and LEG-3. FIG. 19B graphically illustrates the *in vivo* effect of LEG-3 (49.4 μmol/kg) (n=8) compared to Dox (1.72 μmol/kg) (n=8) in the MDA-PCa-2b human prostate carcinoma xenograft model and H&E stained MDA-PCa-2b human prostate carcinoma. FIG. 19C graphically illustrates survival of HT1080 human fibrosarcoma tumors that were treated or not treated with Dox or LEG-3. FIG. 19D graphically illustrates survival of mice bearing MDA-PCa-2b tumors after treatment. FIG. 19E graphically illustrates gross toxicity of tumor bearing mice treated with Dox and LEG-3 as assessed by weight loss and resistance to death.

FIG. 20A-C illustrates that LEG-3 treatment kills tumor-associated macrophages (TAMs) and reduces the level of angiogenic factors in tumors. FIG. 20A shows immunohistochemical stained sections of CT26 tumors from mice that were treated with LEG-3. Legumain stained green and TAMs are identified by CD68 staining, which was red. FIG. 20B provides an example of angiogenic factor arrays. FIG. 20C illustrates the level of angiogenic factors during LEG-3 treatment.

FIG. 21 illustrates the efficacy of the liposome-encapsulated LEG-3 prodrug. Liposome LEG-3 had substantial tumor suppressive effect in 4T1 syngeneic murine breast carcinoma models. LEG-3 was administered in a dosage of 40 umol/kg and liposome-encapsulated legubicin was administered at a dosage level of 5 umol/kg.

Detailed Description of the Invention

The invention provides compositions and methods for targeting agents to cancerous cells and to the tumor microenvironment or primary as well as metastatic tumor sites. In one embodiment, the agent is a prodrug that is substantially non-toxic to normal, noncancerous cells but that is activated on the cell surface of tumor cells and tumor-associated macrophages. The prodrugs of the invention are highly cytotoxic to tumor cells and to tumor-associated cells.

One benefit of the present invention is that prodrugs are processed at the cell surface of tumor cells, and essentially do not enter normal cells or even cancer cells. When the drug or chemotherapeutic agent is cleaved from the prodrug by asparaginyl endopeptidases present on the tumor cell surface (e.g. legumain), the drug or chemotherapeutic agent is released into the tumor microenvironment and can be taken up not only by cancer/tumor cells but also by other tumor-associated cells.

Cancer markers are frequently not expressed by all cancer cells all the time. Thus, release of the drug or chemotherapeutic agent just outside of a tumor cell gives rise to a "bystander" effect upon nearby tumor cells, stromal cells, tumor-asscoiated mcrophages, endothelial cells, vascular cells and other cells that support the growth and development of the tumor. Without such a bystander effect, targeted therapy will often cause tumor cell resistance to the drug or chemotherapeutic agent by "selection" of cancerous tumor cells that do not express the target of the drug or chemotherapeutic agetn. Thus, the prodrugs of the invention are specifically designed to be activated by tumor cell-associated proteases present in the tumor microenvironment and attack all cells in the tumor microenvironment.

According to the invention, the present prodrugs and methods often cause tumor-associated macrophages and endothelial cells to die first because, unlike cancer cells, they are not drug resistant. Tumor-associated macrophages provide angiogenic factors and growth factors. Therefore, the outcome of the present methods is an anti-angiogenic effect that results in tumor cell death by starvation. Data provided herein illustrates that the present prodrugs, agents and methods do inhibit angiogenesis.

The agents used in the prodrugs of the invention may be drugs, cytotoxic agents, chemotherapeutic agents or agents useful for imaging and diagnosis. According to the invention, legumain, a novel asparaginyl endopeptidase, is preferentially expressed in tumors. As shown herein, legumain was detected in membrane-associated vesicles concentrated at the invadopodia of tumor cells, and, unexpectedly, on cell surfaces where it co-localizes with integrins. Cells that over-expressed legumain possessed increased migratory and invasive activity *in vitro*, and adopted an invasive and metastatic phenotype *in vivo*.

Accordingly, legumain may have a role in tumor invasion and metastasis. The invention also provides prodrugs that include a legumain-cleavable peptide linked to a cytotoxic agent. Exemplary prodrug agents, designated legubicin and LEG-3, exhibited reduced toxicity and enhanced tumoricidal activity *in vivo* in a murine colon carcinoma model relative to doxorubicin.

Legumain

Legumain is a lysosomal protease, and a member of the C13 family of cysteine proteases (Chen et al., 1997). Legumain is evolutionarily conserved and is present in plants, invertebrate parasites, as well as in mammals. An example of an amino acid sequence for a preproprotein of a human legumain can be found in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/) at accession number NP 005597 (gi: 21914881), and is reproduced below (SEQ ID NO:1).

```
  1 MVWKVAVFLS VALGIGAIPI DDPEDGGKHW VVIVAGSNGW
 41 YNYRHQADAC HAYQIIHRNG IPDEQIVVMM YDDIAYSEDN
 81 PTPGIVINRP NGTDVYQGVP KDYTGEDVTP QNFLAVLRGD
121 AEAVKGIGSG KVLKSGPQDH VFIYFTDHGS TGILVFPNED
161 LHVKDLNETI HYMYKHKMYR KMVFYIEACE SGSMMNHLPD
201 NINVYATTAA NPRESSYACY YDEKRSTYLG DWYSVNWMED
241 SDVEDLTKET LHKQYHLVKS HTNTSHVMQY GNKTISTMKV
281 MQFQGMKRKA SSPVPLPPVT HLDLTPSPDV PLTIMKRKLM
321 NTNDLEESRQ LTEEIQRHLD ARHLIEKSVR KIVSLLAASE
361 AEVEQLLSER APLTGHSCYP EALLHFRTHC FNWHSPTYEY
401 ALRHLYVLVN LCEKPYPLHR IKLSMDHVCL GHY
```

An example of a nucleotide sequence for a human legumain that encodes SEQ ID NO:1 can found in the NCBI database at accession number NM_005606 (gi: 21914880). This nucleotide sequence is reproduced below (SEQ ID NO:2).

```
  1 GGCACGAGGG AGGCTGCGAG CCGCCGCGAG TTCTCACGGT
 41 CCCGCCGGCG CCACCACCGC GGTCACTCAC CGCCGCCGCC
 81 GCCACCACTG CCACCACGGT CGCCTGCCAC AGGTGTCTGC
121 AATTGAACTC CAAGGTGCAG AATGGTTTGG AAAGTAGCTG
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 161 TATTCCTCAG TGTGGCCCTG GGCATTGGTG CCATTCCTAT
 201 AGATGATCCT GAAGATGGAG GCAAGCACTG GGTGGTGATC
 241 GTGGCAGGTT CAAATGGCTG GTATAATTAT AGGCACCAGG
 281 CAGACGCGTG CCATGCCTAC CAGATCATTC ACCGCAATGG
 321 GATTCCTGAC GAACAGATCG TTGTGATGAT GTACGATGAC
 361 ATTGCTTACT CTGAAGACAA TCCCACTCCA GGAATTGTGA
 401 TCAACAGGCC CAATGGCACA GATGTCTATC AGGGAGTCCC
 441 GAAGGACTAC ACTGGAGAGG ATGTTACCCC ACAAAATTTC
 481 CTTGCTGTGT TGAGAGGCGA TGCAGAAGCA GTGAAGGGCA
 521 TAGGATCCGG CAAAGTCCTG AAGAGTGGCC CCCAGGATCA
 561 CGTGTTCATT TACTTCACTG ACCATGGATC TACTGGAATA
 601 CTGGTTTTTC CCAATGAAGA TCTTCATGTA AAGGACCTGA
 641 ATGAGACCAT CCATTACATG TACAAACACA AAATGTACCG
 681 AAAGATGGTG TTCTACATTG AAGCCTGTGA GTCTGGGTCC
 721 ATGATGAACC ACCTGCCGGA TAACATCAAT GTTTATGCAA
 761 CTACTGCTGC CAACCCCAGA GAGTCGTCCT ACGCCTGTTA
 801 CTATGATGAG AAGAGGTCCA CGTACCTGGG GGACTGGTAC
 841 AGCGTCAACT GGATGGAAGA CTCGGACGTG GAAGATCTGA
 881 CTAAAGAGAC CCTGCACAAG CAGTACCACC TGGTAAAATC
 921 GCACACCAAC ACCAGCCACG TCATGCAGTA TGGAAACAAA
 961 ACAATCTCCA CCATGAAAGT GATGCAGTTT CAGGGTATGA
1001 AACGCAAAGC CAGTTCTCCC GTCCCCCTAC CTCCAGTCAC
1041 ACACCTTGAC CTCACCCCCA GCCCTGATGT GCCTCTCACC
1081 ATCATGAAAA GGAAACTGAT GAACACCAAT GATCTGGAGG
1121 AGTCCAGGCA GCTCACGGAG GAGATCCAGC GGCATCTGGA
1161 TGCCAGGCAC CTCATTGAGA AGTCAGTGCG TAAGATCGTC
1201 TCCTTGCTGG CAGCGTCCGA GGCTGAGGTG GAGCAGCTCC
1241 TGTCCGAGAG AGCCCCGCTC ACGGGGCACA GCTGCTACCC
1281 AGAGGCCCTG CTGCACTTCC GGACCCACTG CTTCAACTGG
1321 CACTCCCCCA CGTACGAGTA TGCGTTGAGA CATTTGTACG
1361 TGCTGGTCAA CCTTTGTGAG AAGCCGTATC CACTTCACAG
1401 GATAAAATTG TCCATGGACC ACGTGTGCCT TGGTCACTAC
1441 TGAAGAGCTG CCTCCTGGAA GCTTTTCCAA GTGTGAGCGC
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1481 CCCACCGACT GTGTGCTGAT CAGAGACTGG AGAGGTGGAG
1521 TGAGAAGTCT CCGCTGCTCG GGCCCTCCTG GGGAGCCCCC
1561 GCTCCAGGGC TCGCTCCAGG ACCTTCTTCA CAAGATGACT
1601 TGCTCGCTGT TACCTGCTTC CCCAGTCTTT TCTGAAAAAC
1641 TACAAATTAG GGTGGGAAAA GCTCTGTATT GAGAAGGGTC
1681 ATATTTGCTT TCTAGGAGGT TTGTTGTTTT GCCTGTTAGT
1721 TTTGAGGAGC AGGAAGCTCA TGGGGGCTTC TGTAGCCCCT
1761 CTCAAAAGGA GTCTTTATTC TGAGAATTTG AAGCTGAAAC
1801 CTCTTTAAAT CTTCAGAATG ATTTTATTGA AGAGGGCCGC
1841 AAGCCCCAAA TGGAAAACTG TTTTTAGAAA ATATGATGAT
1881 TTTTGATTGC TTTTGTATTT AATTCTGCAG GTGTTCAAGT
1921 CTTAAAAAAT AAAGATTTAT AACAGAACCC AAAAAAAAAA
1961 AAAAAAAAAA AAAAAAAAAA A
```

A genomic nucleotide sequence for human legumain can be found in the NCBI database at accession number NT 026437 (gi: 29736559). *See* website at ncbi.nlm.nih.gov. Naturally occurring allelic variants of legumain nucleic acids and proteins are also contemplated. An allelic variant is an alternate form of sequence which may have a substitution, deletion or addition at one or more positions, which does not substantially alter the function of the legumain.

Murine legumain shares about 83% sequence identity with the human protein (Barrett et al., 2001). An amino acid sequence for murine legumain can be found in the NCBI database at accession number O89017 (gi: 21617821).

Importantly, legumain has a highly restricted protease specificity. In particular, legumain cleaves polypeptide sequences on the N-terminal side of asparagine. Hence, legumain requires an asparagine at the P1 site of a substrate in order to cleave a protein or peptide.

Mammalian legumain has been implicated in processing of bacterial peptides and in processing endogenous proteins for MHC class II presentation in the lysosomal/endosomal systems (Manoury et al., 1998; Beck et al., 2001). Recently, human legumain has been identified as an inhibitor of osteoclast formation and has been associated with bone resorption (Choi et al., 2001). However, elucidation of legumain's functional role in molecular cell biology and

pathobiology is limited, and association with tumor biology has not previously been demonstrated or suggested.

It is herein disclosed that cancerous tissues express legumain. Legumain expression is also correlated with a propensity for cell migration and cancer cell metastasis. In some embodiments, the legumain is expressed on the surface of the cancerous cells in which it is expressed.

The invention provides therapeutic and diagnostic compositions of prodrugs and other agents that can be targeted to tissues having cancerous cells. Some of the prodrugs and targeted agents of the invention contain a peptide that has an amino acid sequence that can be recognized, bound or cleaved by legumain. In other embodiments, the invention provides agents that can bind legumain, for example, legumain inhibitors and antibodies that recognize and bind legumain.

Prodrugs

In some embodiments of the invention, agents are designed to contain a drug compound that is activated following cleavage by asparaginyl endopeptidase, for example, legumain. These agents are referred to herein as prodrugs.

Hence, the invention relates to a prodrug compound, including a drug molecule linked to a peptide, wherein the peptide has an amino acid sequence including two linked amino acids, wherein at least one of the two linked amino acids is Asn, and wherein legumain cleaves the peptide at the link between the two amino acids to generate an active drug from the prodrug. The term "drug" as used herein, refers to any medicinal substance used in humans or other animals. Encompassed within this definition are chemotherapeutic agents, cytotoxic agents, compound analogs, hormones, antimicrobials, neurotransmitters, etc. In some embodiments, the prodrugs of the invention include drug molecules whose activity is diminished when attached to peptide.

For example, in some embodiments the prodrug can have a structure similar to that provided for SEQ ID NO:3:

$$\text{Hyd-(Xaa1)}_n\text{-Xaa2-Asn-(Xaa3)-drug}$$

wherein:

Hyd is a hydrophilic protecting group;

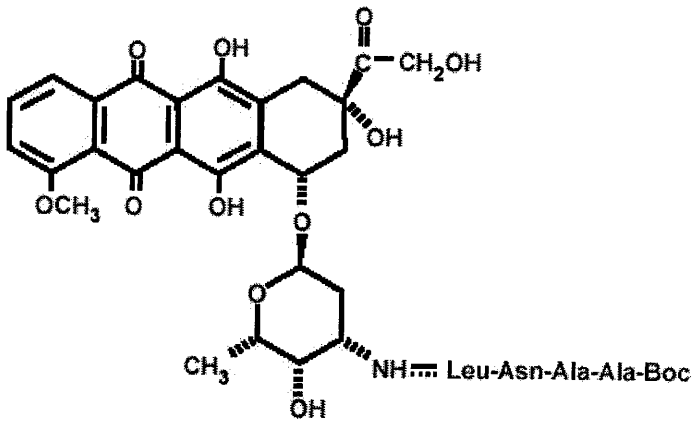
n is an integer of about 0 to about 50;

Xaa1 and Xaa2 are separately any amino acid;

Xaa3 is either nothing or an amino acid that has no substantial effect on the activity of the drug; and

the drug employed is a drug whose action is diminished or blocked by attachment of a peptide to the drug.

In one embodiment, the invention provides legubicin as a prodrug of the invention that contains a cytotoxic agent, doxorubicin, linked to a peptidyl sequence (Boc-Ala-Ala-Asn-Leu, SEQ ID NO:4). The structure of legubicin is provided below as formula IA.

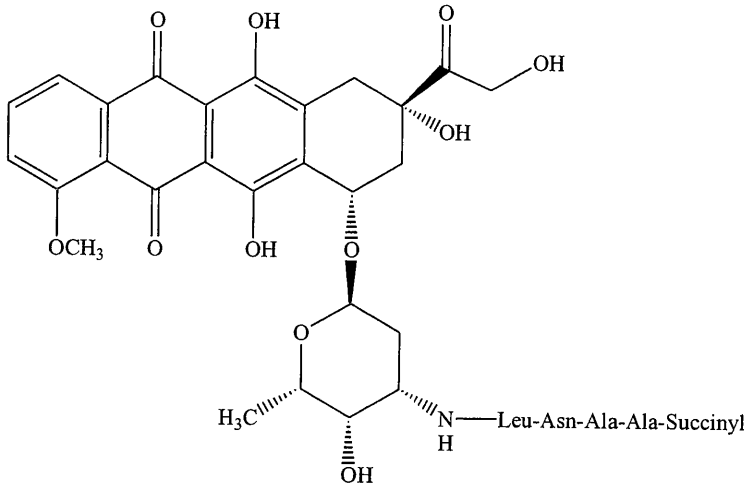


Note that in legubicin, an amino group in doxorubicin is attached to the C-terminus of the peptide Boc-Ala-Ala-Asn-Leu (SEQ ID NO:4).

Intact legubicin is not significantly cytotoxic. However, legubicin becomes toxic after the amino acid sequence of the linked peptide (e.g., Boc-Ala-Ala-Asn-Leu (SEQ ID NO:4)) is cleaved by legumain. Legumain can cleave legubicin between the leucine and the asparagine of SEQ ID NO:4, thereby releasing doxorubicin-Leu to act as a cytotoxin on the cells that express legumain.

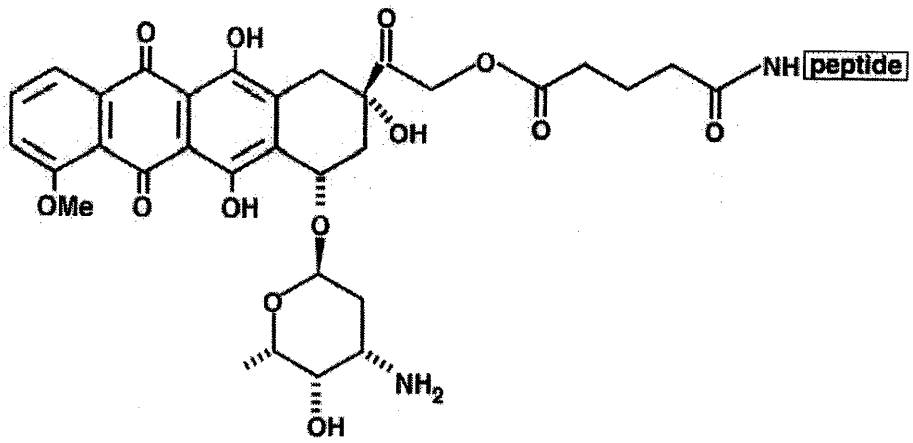
In another embodiment, the invention provides LEG-3 as a prodrug of the invention that contains the cytotoxic agent, doxorubicin, linked to a peptidyl

sequence succinyl-Ala-Ala-Asn-Leu-doxorubicin (SEQ ID NO:8). The structure of LEG-3 is shown below as formula IB.



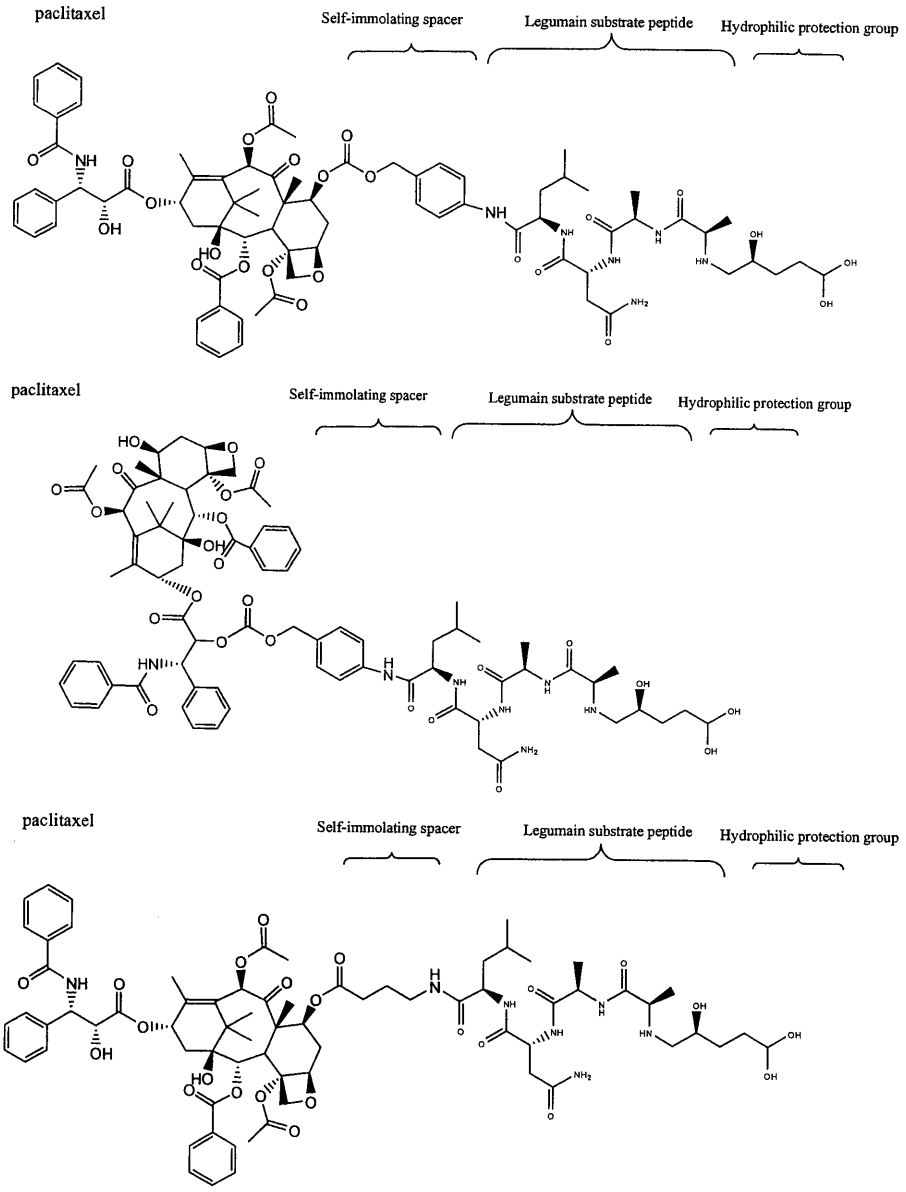
LEG-3 is essentially non-toxic to normal, non-cancerous cells that do not express asparaginyl endopeptidases. One beneficial characteristic of LEG-3 is that it is water-soluble and does not penetrate cell membranes or accumulate in normal, non-cancerous tissues. The succinyl group of LEG-3 tends to make LEG-3 more hydrophilic than legubicin. Accordingly, LEG-3 has even less toxicity than legubicin.

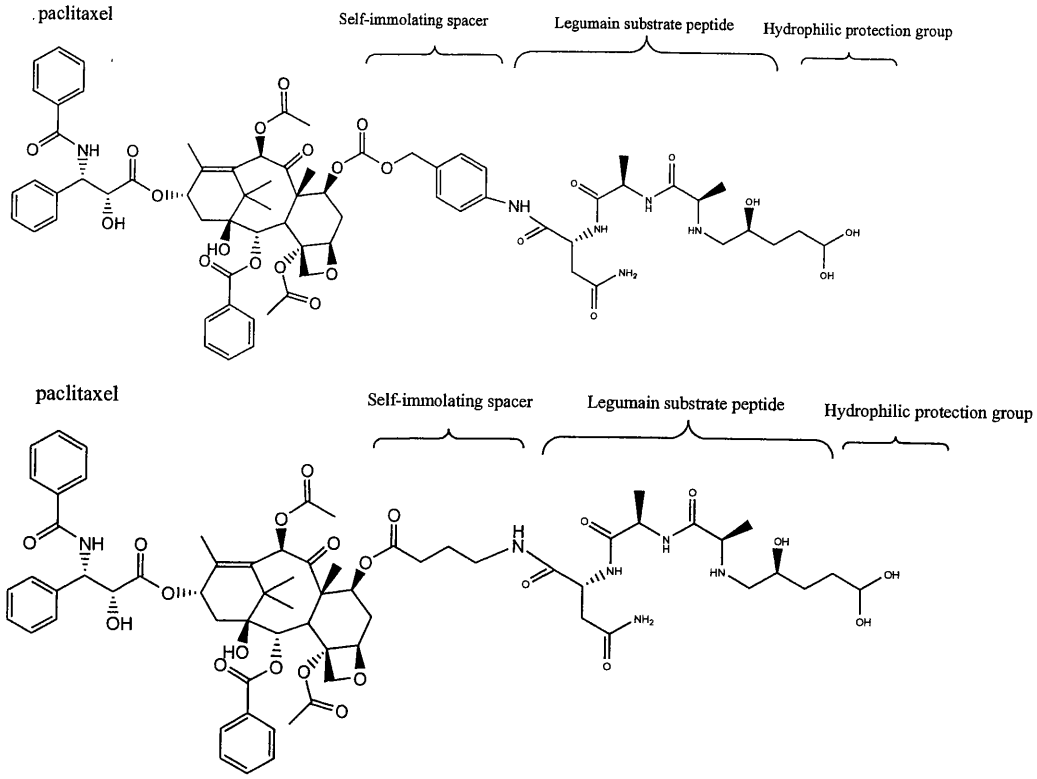
In general, while a peptide could be linked to the $-CO-CH_2-OH$ moiety of doxorubicin to generate a prodrug having formula II, such a prodrug construct is less desirable than a doxorubicin prodrug having a linkage at the amino position shown in formula IA or IB. For example, a less desirable doxorubicin prodrug having formula II is shown below.



While doxorubicin prodrugs having formula II can be properly targeted to legumain-expressing cells, such prodrugs appear to more toxic than prodrugs having linkages like those shown in formulae IA and IB. Hence, linkage of peptides to the heterocyclic ring of doxorubicin is preferred.

Prodrugs that include paclitaxel are also highly desirable and can have any of the following structures.





As provided herein, peptides linked to drugs can have a variety of sequences and a variety of lengths to form the prodrugs of the invention. Hence, any peptide can be used so long as the peptide sequence contains Asn at a position sufficiently near the drug that the drug is active after cleavage of the prodrug. Generally, peptides are used that block or inhibit some or all of the drug's activity. In some embodiments, the linked peptide can have an amino acid sequence that includes the sequence Asn-Leu. In other embodiments, the peptide can have an amino acid sequence that includes the sequence Ala-Asn-Leu. Further embodiments can have peptides with an amino acid sequence that include the sequence Thr-Asn-Leu, or the sequence Ala-Ala-Asn-Leu (SEQ ID NO:5), or the sequence Ala-Thr-Asn-Leu (SEQ ID NO:6). In some embodiments, the peptide further can have an *N*-β-alanyl terminus, an N-terminal Boc or an N-terminal succinyl residue.

Specific examples of doxorubicin prodrugs contemplated by the invention include the following:

Boc-Ala-Ala-Asn-Leu-doxorubicin (SEQ ID NO:7).

Succinyl-Ala-Ala-Asn-Leu-doxorubicin (SEQ ID NO:8).

Boc-Ala-Thr-Asn-Leu-doxorubicin (SEQ ID NO:9).

Succinyl-Ala-Thr-Asn-Leu-doxorubicin (SEQ ID NO:10).

Boc-Ala-Asn-Leu-doxorubicin (SEQ ID NO:11).

Succinyl-Ala-Asn-Leu-doxorubicin (SEQ ID NO:12).

Boc-Thr-Leu-doxorubicin (SEQ ID NO:13).

Succinyl-Thr-Leu-doxorubicin (SEQ ID NO:14).

In other embodiments of the invention, agents containing a detection agent are targeted to legumain-containing tissues for detection and diagnosis of cancer, cell migration or metastasis. Such diagnostic agents can include an agent that binds to legumain and a detectable label or reporter molecule. For example, such a diagnostic agent can be a legumain inhibitor or an anti-legumain antibody, that specifically recognizes or binds to legumain, and that has a label linked to it. These agents are useful for imaging, diagnosis, and for treating cancer and are discussed in more detail below.

In many embodiments, the agents and prodrugs of the invention are substantially non-immunogenic to the animal to which they are administered. The term "substantially non-immunogenic" means that the agent or prodrug can be administered to the animal on more than one occasion without causing a significant immune response. Such a significant immune response can be seen, for example, if a foreign protein or an antibody from another species were administered to the animal and a significant humoral or cellular immune response was initiated.

As described above, the prodrugs and diagnostic agents of the invention can include a peptide. For example, a peptide can be linked to a cytotoxic agent to modulate the cytotoxicity of the cytotoxic agent. In other embodiments, a peptide can be linked to a drug to modulate the activity of the drug. In other embodiments, a diagnostic agent can include a peptide that links a label or a reporter molecule to a an agent that binds to legumain. An agent that binds to legumain can be a peptide, for example, agents that bind to legumain include peptide substrates and peptide inhibitors of legumain. Other agents that bind to legumain include anti-legumain antibodies.

The peptides employed can have amino acid sequences comprised of any available amino acid, although in some embodiment the peptide has an

asparagine residue at a desired cleavage site. Amino acids included in the peptides can be genetically encoded L-amino acids, naturally occurring non-genetically encoded L-amino acids, synthetic L-amino acids or D-enantiomers of any of the above. The amino acid notations used herein for the twenty genetically encoded L-amino acids and common non-encoded amino acids are conventional and are as shown in Table 1. These amino acids can be linked together, for example, by peptidyl linkages, intersubunit linkages, or other intersubunit linkages that are consistent with enzyme-substrate or receptor-ligand binding interactions.

Table 1

Amino Acid	**One-Letter Symbol**	**Common Abbreviation**
Alanine	A	Ala
Arginine	R	Arg
Asparagine	N	Asn
Aspartic acid	D	Asp
Cysteine	C	Cys
Glutamine	Q	Gln
Glutamic acid	E	Glu
Glycine	G	Gly
Histidine	H	His
Isoleucine	I	Ile
Leucine	L	Leu
Lysine	K	Lys
Methionine	M	Met
Phenylalanine	F	Phe
Proline	P	Pro
Serine	S	Ser
Threonine	T	Thr
Tryptophan	W	Trp
Tyrosine	Y	Tyr
Valine	V	Val
β-Alanine		bAla

Amino Acid	One-Letter Symbol	Common Abbreviation
2,3-Diaminopropionic acid		Dpr
α-Aminoisobutyric acid		Aib
N-Methylglycine (sarcosine)		MeGly
Ornithine		Orn
Citrulline		Cit
t-Butylalanine		t-BuA
t-Butylglycine		t-BuG
N-methylisoleucine		MeIle
Phenylglycine		Phg
Cyclohexylalanine		Cha
Norleucine		Nle
Naphthylalanine		Nal
Pyridylalanine		
3-Benzothienyl alanine		
4-Chlorophenylalanine		Phe(4-Cl)
2-Fluorophenylalanine		Phe(2-F)
3-Fluorophenylalanine		Phe(3-F)
4-Fluorophenylalanine		Phe(4-F)
Penicillamine		Pen
1,2,3,4-Tetrahydro-isoquinoline-3-carboxylic acid		Tic
β-2-thienylalanine		Thi
Methionine sulfoxide		MSO
Homoarginine		hArg
N-acetyl lysine		AcLys
2,4-Diamino butyric acid		Dbu
ρ-Aminophenylalanine		Phe(pNH_2)

Amino Acid	One-Letter Symbol	Common Abbreviation
N-methylvaline		MeVal
Homocysteine		hCys
Homoserine		hSer
ε-Amino hexanoic acid		Aha
δ-Amino valeric acid		Ava
2,3-Diaminobutyric acid		Dab

Certain amino acids which are not genetically encoded and which can be present in agents of the invention include, but are not limited to, β-alanine (b-Ala) and other omega-amino acids such as 3-aminopropionic acid (Dap), 2,3-diaminopropionic acid (Dpr), 4-aminobutyric acid and so forth; α-aminoisobutyric acid (Aib); ε-aminohexanoic acid (Aha); δ-aminovaleric acid (Ava); N-methylglycine (MeGly); ornithine (Orn); citrulline (Cit); t-butylalanine (t-BuA); t-butylglycine (t-BuG); N-methylisoleucine (MeIle); phenylglycine (Phg); cyclohexylalanine (Cha); norleucine (Nle); 2-naphthylalanine (2-Nal); 4-chlorophenylalanine (Phe(4-Cl)); 2-fluorophenylalanine (Phe(2-F)); 3-fluorophenylalanine (Phe(3-F)); 4-fluorophenylalanine (Phe(4-F)); penicillamine (Pen); 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic); .beta.-2-thienylalanine (Thi); methionine sulfoxide (MSO); homoarginine (hArg); N-acetyl lysine (AcLys); 2,3-diaminobutyric acid (Dab); 2,3-diaminobutyric acid (Dbu); p-aminophenylalanine (Phe(pNH_2)); N-methyl valine (MeVal); homocysteine (hCys) and homoserine (hSer). These amino acids also fall into the categories defined above.

The classifications of the above-described genetically encoded and non-encoded amino acids are summarized in Table 2, below. It is to be understood that Table 2 is for illustrative purposes only and does not purport to be an exhaustive list of amino acid residues which may comprise the peptides and peptide analogues described herein. Other amino acid residues which are useful for making the peptides and peptide analogues described herein can be found, e.g., in Fasman, 1989, CRC Practical Handbook of Biochemistry and Molecular Biology, CRC Press, Inc., and the references cited therein. Amino acids not specifically mentioned herein can be conveniently classified into the above-

described categories on the basis of known behavior and/or their characteristic chemical and/or physical properties as compared with amino acids specifically identified.

TABLE 2

Classification	Genetically Encoded	Genetically Non-Encoded
Hydrophobic		
Aromatic	F, Y, W	Phg, Nal, Thi, Tic, Phe(4-Cl), Phe(2-F), Phe(3-F), Phe(4-F), Pyridyl Ala, Benzothienyl Ala
Apolar	M, G, P	
Aliphatic	A, V, L, I	t-BuA, t-BuG, MeIle, Nle, MeVal, Cha, bAla, MeGly, Aib
Hydrophilic		
Acidic	D, E	
Basic	H, K, R	Dpr, Orn, hArg, Phe(p-NH_2), DBU, A_2 BU
Polar	Q, N, S, T, Y	Cit, AcLys, MSO, hSer
Cysteine-Like	C	Pen, hCys, β-methyl Cys

Peptides described herein may be synthesized by methods available in the art, including recombinant DNA methods and chemical synthesis. Chemical synthesis may generally be performed using standard solution phase or solid phase peptide synthesis techniques, in which a peptide linkage occurs through the direct condensation of the α-amino group of one amino acid with the carboxy group of the other amino acid with the elimination of a water molecule. Peptide bond synthesis by direct condensation, as formulated above, requires suppression of the reactive character of the amino group of the first and of the carboxyl group of the second amino acid. The masking substituents must permit their ready removal, without inducing breakdown of the labile peptide molecule.

In solution phase synthesis, a wide variety of coupling methods and protecting groups may be used (see Gross and Meienhofer, eds., "The Peptides: Analysis, Synthesis, Biology," Vol. 1-4 (Academic Press, 1979); Bodansky and Bodansky, "The Practice of Peptide Synthesis," 2d ed. (Springer Verlag, 1994)). In addition, intermediate purification and linear scale up are possible. Those of ordinary skill in the art will appreciate that solution synthesis requires consideration of main chain and side chain protecting groups and activation method. In addition, careful segment selection may be necessary to minimize racemization during segment condensation. Solubility considerations are also a factor.

Solid phase peptide synthesis uses an insoluble polymer for support during organic synthesis. The polymer-supported peptide chain permits the use of simple washing and filtration steps instead of laborious purifications at intermediate steps. Solid-phase peptide synthesis may generally be performed according to the method of Merrifield et al., J. Am. Chem. Soc. 85:2149, 1963, which involves assembling a linear peptide chain on a resin support using protected amino acids. Solid phase peptide synthesis typically utilizes either the Boc or Fmoc strategy, which are now well known in the art.

Those of ordinary skill in the art will recognize that, in solid phase synthesis, deprotection and coupling reactions must go to completion and the side-chain blocking groups must be stable throughout the entire synthesis. In addition, solid phase synthesis is generally most suitable when peptides are to be made on a small scale.

Drugs

According to the invention, any drug useful for modulating, treating or otherwise affecting the physiological state of a legumain-expressing cell can be attached to a legumain peptide substrate or an agent that can bind legumain. Agents that can bind legumain include legumain inhibitors, anti-legumain antibodies and the like. Attachment of drugs to legumain peptide substrates generates a prodrug of the invention. Similarly, attachment of a drug to an agent that can bind legumain permits delivery and accumulation of the drug in legumain-expressing cells.

While the invention is directed to attaching any drug to a legumain peptide substrate or an agent that can bind legumain, in some embodiments, the drug is a cytotoxic agent or an effector molecule. The cytotoxic agents and effector molecules useful in the practice of the invention include cytotoxins and chemotherapeutic agents. These agents include, but are not limited to, folate antagonists, pyrimidine antimetabolites, purine antimetabolites, alkylating agents, platinum anti-tumor agents, anthracyclines, DNA intercalators, epipodophyllotoxins, DNA topoisomerases, microtubule-targeting agents, vinca alkaloids, taxanes, epothilones and asparaginases. Further information can be found in Bast et al., CANCER MEDICINE, edition 5, which is available free as a digital book. See website at ncbi.nlm.nih.gov/books/bv.fcgi?call=bv View..ShowTOC&rid=cmed. TOC&depth=2.

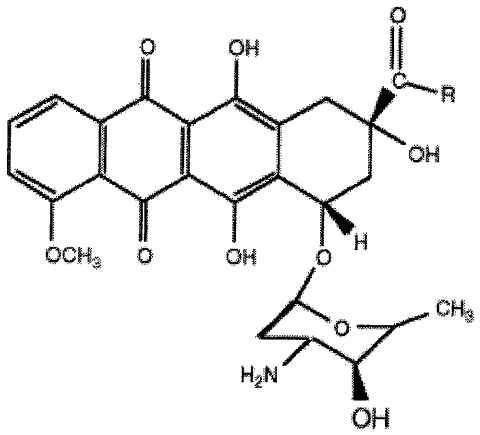
Folic acid antagonists are cytotoxic drugs used as antineoplastic, antimicrobial, anti-inflammatory, and immune-suppressive agents. While several folate antagonists have been developed, and several are now in clinical trial, methotrexate (MTX) is the antifolate with the most extensive history and widest spectrum of use. MTX is an essential drug in the chemotherapy regimens used to treat patients with acute lymphoblastic leukemia, lymphoma, osteosarcoma, breast cancer, choriocarcinoma, and head and neck cancer, as well as being an important agent in the therapy of patients with nonmalignant diseases, such as rheumatoid arthritis, psoriasis, and graft-versus-host disease.

Pyrimidine antimetabolites include fluorouracil, cytosine arabinoside, 5-azacytidine, and 2', 2'-difluoro-2'-deoxycytidine. Purine antimetabolites include 6-mercatopurine, thioguanine, allopurinol (4-hydroxypyrazolo-3,4-*d*-pyrimidine), deoxycoformycin (pentostatin), 2-fluoroadenosine arabinoside (fludarabine; 9-ß-d-arabinofuranosyl-2-fluoradenine), and 2-chlorodeoxyadenosine (Cl-dAdo, cladribine). In addition to purine and pyrimidine analogues, other agents have been developed that inhibit biosynthetic reactions leading to the ultimate nucleic acid precursors. These include phosphonacetyl-L-aspartic acid (PALA), brequinar, acivicin, and hydroxyurea.

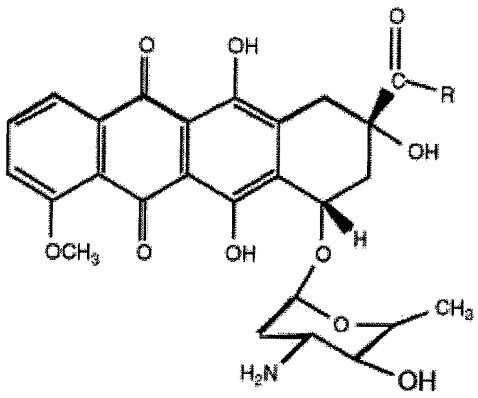
Alkylating agents and the platinum anti-tumor compounds form strong chemical bonds with electron-rich atoms (nucleophiles), such as sulfur in proteins and nitrogen in DNA. Although these compounds react with many

biologic molecules, the primary cytotoxic actions of both classes of agents appear to be the inhibition of DNA replication and cell division produced by their reactions with DNA. However, the chemical differences between these two classes of agents produce significant differences in their anti-tumor and toxic effects. The most frequently used alkylating agents are the nitrogen mustards. Although thousands of nitrogen mustards have been synthesized and tested, only five are commonly used in cancer therapy today. These are mechlorethamine (the original "nitrogen mustard"), cyclophosphamide, ifosfamide, melphalan, and chlorambucil. Closely related to the nitrogen mustards are the aziridines, which are represented in current therapy by thiotepa, mitomycin C, and diaziquone (AZQ). Thiotepa (triethylene thiophosphoramide) has been used in the treatment of carcinomas of the ovary and breast and for the intrathecal therapy of meningeal carcinomatosis. The alkyl alkane sulfonate, busulfan, was one of the earliest alkylating agents. This compound is one of the few currently used agents that clearly alkylate through an SN2 reaction. Hepsulfam, an alkyl sulfamate analogue of busulfan with a wider range of anti-tumor activity in preclinical studies, has been evaluated in clinical trials but thus far has demonstrated no superiority to busulfan. Busulfan has a most interesting, but poorly understood, selective toxicity for early myeloid precursors. This selective effect is probably responsible for its activity against chronic myelocytic leukemia (CML).

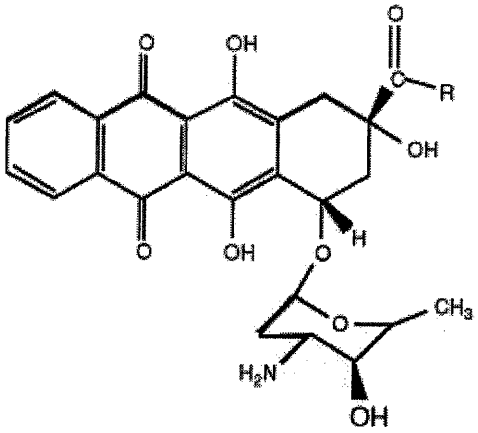
Topoisomerase poisons are believed to bind to DNA, the topoisomerase, or either molecule at or near the region of the enzyme involved in the formation of the DNA protein covalent linkage. Many topoisomerase poisons, such as the anthracyclines and actinomycin D, are relatively planar hydrophobic compounds that bind to DNA with high affinity by intercalation, which involves stacking of the compound between adjacent base pairs. Anthracyclines intercalate into double-stranded DNA and produce structural changes that interfere with DNA and RNA syntheses. Several of the clinically relevant anthracyclines are shown below.



	R
Doxorubicin	OH
Daunorubicin	H



Epirubicin

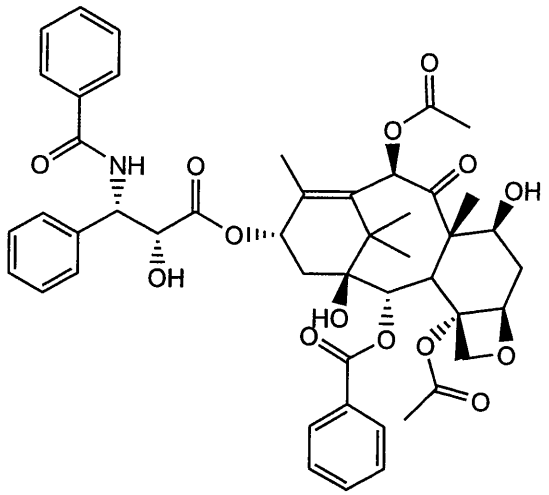


Idarabicin

Non-intercalating topoisomerase-targeting drugs include epipodophyllotoxins such as etoposide and teniposide. Etoposide is approved in the United States for the treatment of testicular and small cell lung carcinomas. Etoposide phosphate is more water soluble than etoposide and is rapidly

converted to etoposide in vivo. Other non-intercalating topoisomerase-targeting drugs include topotecan and irinotecan.

Unique classes of natural product anticancer drugs have been derived from plants. As distinct from those agents derived from bacterial and fungal sources, the plant products, represented by the *Vinca* and *Colchicum* alkaloids, as well as other plant-derived products such as paclitaxel (Taxol) and podophyllotoxin, do not target DNA. Rather, they either interact with intact microtubules, integral components of the cytoskeleton of the cell, or with their subunit molecules, the tubulins. Clinically useful plant products that target microtubules include the *Vinca* alkaloids, primarily vinblastine (VLB), vincristine (VCR), vinorelbine (Navelbine, VRLB), and a newer *Vinca* alkaloid, vinflunine (VFL; 20',20'-difluoro-3',4'-dihydrovinorelbine), as well as the two taxanes, paclitaxel and docetaxel (Taxotere). The structure of paclitaxel is provided below.



Hence, examples of drugs that can be used to form prodrugs of the invention include, but are not limited to, Aldesleukin, Asparaginase, Bleomycin Sulfate, Camptothecin, Carboplatin, Carmustine, Cisplatin, Cladribine, Cyclophosphamide (lyophilized), Cyclophosphamide (non-lyophilized), Cytarabine (lyophilized powder), Dacarbazine, Dactinomycin, Daunorubicin, Diethyistilbestrol, Doxorubicin, Epoetin Alfa, Esperamycin, Etidronate, Etoposide, Filgrastim, Floxuridine, Fludarabine Phosphate, Fluorouracil, Goserelin, Granisetron Hydrochloride, Idarubicin, Ifosfamide, Immune Globulin, Interferon, Alpha-2a, Interferon Alpha-2b, Leucovorin Calcium, Leuprolide,

Levamisole, Mechiorethamine, Medroxyprogesterone, Melphalan, Methotrexate, Mitomycin, Mitoxantrone, Octreotide, Ondansetron Hydrochloride, Paclitaxel, Pamidronate, Disodium, Pegaspargase, Plicamycin, Sargramostim, Streptozocin, Taxol, Thiotepa, Teniposide, Vinblastine, and Vincristine. Other toxic effector molecules for use in the present invention are disclosed, for example, in WO 98/13059; Payne, 2003; US 2002/0147138 and other references available to one of skill in the art.

Peptides can be conjugated to chemotherapeutic agents, drugs, reporter molecules, labels, cytotoxic agents and other entities by using peptidyl amino groups, carboxylate groups or side chain moieties of the peptidyl amino acids to form covalent linkages with such chemotherapeutic agents, drugs, reporter molecules, labels, cytotoxic agents and other agents. Amino acids can be conjugated to such entities by any method available to one of skill in the art. For example, functional groups present on the side chains of amino acids in the peptides can be combined with functional groups in the entity to which the peptide is conjugated. Functional groups that can form covalent bonds include, for example, --COOH and --OH; --COOH and --NH_2; and --COOH and --SH. Pairs of amino acids that can be used to conjugate proteins to the present peptides include, Asp and Lys; Glu and Lys; Asp and Arg; Glu and Arg; Asp and Ser; Glu and Ser; Asp and Thr; Glu and Thr; Asp and Cys; and Glu and Cys. Other examples of amino acid residues that are capable of forming covalent linkages with one another include cysteine-like amino acids such Cys, hCys, β-methyl-Cys and Pen, which can form disulfide bridges with one another. Other pairs of amino acids that can be used for conjugation and cyclization of the peptide will be apparent to those skilled in the art.

The groups used to conjugate a peptide to another agent need not be a side group on an amino acid. Examples of functional groups capable of forming a covalent linkage with the amino terminus of a peptide include, for example, carboxylic acids and esters. Examples of functional groups capable of forming a covalent linkage with the carboxyl terminus of a peptide include --OH, --SH, --NH_2 and --NHR where R is (C_1 - C_6) alkyl, (C_1 - C_6) alkenyl and (C_1 - C_6) alkynyl.

The variety of reactions between two side chains with functional groups suitable for forming such linkages, as well as reaction conditions suitable for forming such linkages, will be apparent to those of skill in the art. Preferably, the reaction conditions used to conjugate the peptides to other entities are sufficiently mild so as not to degrade or otherwise damage the peptide. In particular, some embodiments require a functional legumain recognition site (e.g. an intact asparagine), so conditions should be adjusted to minimize damage to such sites. Suitable groups for protecting the various functionalities as necessary are well known in the art (see, e.g., Greene & Wuts, 1991, 2nd ed., John Wiley & Sons, NY), as are various reaction schemes for preparing such protected molecules.

Methods for linking peptides to other entities are available in the art. See Spatola, 1983, "Peptide Backbone Modifications" In: Chemistry and Biochemistry of Amino Acids Peptides and Proteins (Weinstein, ed.), Marcel Dekker, New York, p. 267 (general review); Morley, 1980, Trends Pharm. Sci. 1:463-468; Hudson et al., 1979, Int. J. Prot. Res. 14:177-185 (--CH_2 NH--, --CH_2 CH_2 --); Spatola et al., 1986, Life Sci. 38:1243-1249 (--CH_2 --S); Hann, 1982, J. Chem. Soc. Perkin Trans. I. 1:307-314 (--CH = CH--, cis and trans); Almquist et al., 1980, J. Med. Chem. 23:1392-1398 (--CO CH_2 --); Jennings-White et al., Tetrahedron. Lett. 23:2533 (--CO CH_2--); European Patent Application EP 45665 (1982) CA:97:39405 (--CH(OH) CH_2 --); Holladay et al., 1983, Tetrahedron Lett. 24:4401-4404 (--C(OH)CH_2--); and Hruby, 1982, Life Sci. 31:189-199 (--CH_2 --S--).

Cancer Treatment

In certain aspects of the inventions, the prodrug compounds and agents described herein are useful for preventing, treating or diagnosing cancer. As used herein, the term "cancer" includes solid mammalian tumors as well as hematological malignancies.

"Solid mammalian tumors" include cancers of the head and neck, lung, mesothelioma, mediastinum, esophagus, stomach, pancreas, hepatobiliary system, small intestine, colon, colorectal, rectum, anus, kidney, urethra, bladder, prostate, urethra, penis, testis, gynecological organs, ovaries, breast, endocrine

system, skin central nervous system; sarcomas of the soft tissue and bone; and melanoma of cutaneous and intraocular origin.

The term "hematological malignancies" includes childhood leukemia and lymphomas, Hodgkin's disease, lymphomas of lymphocytic and cutaneous origin, acute and chronic leukemia, plasma cell neoplasm and cancers associated with AIDS.

In addition, a cancer at any stage of progression can be treated, such as primary, metastatic, and recurrent cancers. The invention can also be used to treat autoimmune deficiency syndrome-associated Kaposi's sarcoma, cancer of the adrenal cortex, cancer of the cervix, cancer of the endometrium, cancer of the esophagus, cancer of the head and neck, cancer of the liver, cancer of the pancreas, cancer of the prostate, cancer of the thymus, carcinoid tumors, chronic lymphocytic leukemia, Ewing's sarcoma, gestational trophoblastic tumors, hepatoblastoma, multiple myeloma, non-small cell lung cancer, retinoblastoma, or tumors in the ovaries. A cancer at any stage of progression can be treated or detected, such as primary, metastatic, and recurrent cancers. Information regarding numerous types of cancer can be found, *e.g.*, from the American Cancer Society (www.cancer.org), or from, *e.g.*, Wilson et al. (1991) Harrison's Principles of Internal Medicine, 12th Edition, McGraw-Hill, Inc. Both human and veterinary uses are contemplated.

As used herein the terms "normal mammalian cell" and "normal animal cell" are defined as a cell that is growing under normal growth control mechanisms (*e.g.*, genetic control) and that displays normal cellular differentiation and normal migration patterns. Cancer cells differ from normal cells in their growth patterns, migration and in the nature of their cell surfaces. For example cancer cells tend to grow continuously and chaotically, without regard for their neighbors, and can sometimes migrate to distal sites to generate tumors in other areas of the body.

The present invention is directed, in some embodiments, to methods of treating cancer in an animal, for example, for human and veterinary uses, which include administering to a subject animal (e.g., a human), a therapeutically effective amount of an agent (e.g. a prodrug or a legumain inhibitor) of the present invention.

Treatment of, or treating, cancer is intended to include the alleviation of or diminishment of at least one symptom typically associated with the disease. The treatment also includes alleviation or diminishment of more than one symptom. The treatment may cure the cancer, e.g., it may substantially kill the cancer cells and/or it may arrest or inhibit the growth of the cancerous tumor.

Anti-cancer activity can be evaluated against varieties of cancers using methods available to one of skill in the art. Anti-cancer activity, for example, is determined by identifying the lethal dose (LD100) or the 50% effective dose (ED50) or the dose that inhibits growth at 50% (GI50) of an agent of the present invention that prevents the growth of a cancer. In one aspect, anti-cancer activity is the amount of the agents that kills 50% or 100% of the cancer cells, for example, when measured using standard dose response methods.

The present invention also provides a method of evaluating a therapeutically effective dosage for treating a cancer with an agent of the invention that includes determining the LD100 or ED50 of the agent *in vitro*. Such a method permits calculation of the approximate amount of agent needed per volume to inhibit cancer cell growth or to kill 50% to 100% of the cancer cells. Such amounts can be determined, for example, by standard microdilution methods.

In some embodiments, the agents of the invention can be administered in multiple doses over a period of one to seven days.

The term "animal," as used herein, refers to an animal, such as a warm-blooded animal, which is susceptible to or has a disease associated with legumain expression, for example, cancer. Mammals include cattle, buffalo, sheep, goats, pigs, horses, dogs, cats, rats, rabbits, mice, and humans. Also included are other livestock, domesticated animals and captive animals. The term "farm animals" includes chickens, turkeys, fish, and other farmed animals. Mammals and other animals including birds may be treated by the methods and compositions described and claimed herein.

Other Therapeutic Methods

In addition to expression in cancer cells, legumain is expressed in monocytes. Such monocyte expression is differentially regulated by GM-CSF

and M-CSF. Legumain may also be involved in monocyte or macrophage migration or infiltration, and in antigen processing. Recently, legumain, has been identified as an inhibitor of osteoclast formation and is associated with bone resorption. Choi, S.J., et al., *Osteoclast inhibitory peptide 2 inhibits osteoclast formation via its C-terminal fragment.* J Bone Miner Res, 2001. **16**(10): p. 1804-11.

Accordingly, the invention also contemplates inhibiting osteoclast activity, for example, to prevent and treat osteoporosis. Methods for inhibiting osteoclast activity or for preventing or treating osteoporosis in an animal involve administering to the animal an agent that inhibits legumain. Any inhibitor of legumain can be utilized, for example, any of the inhibitors described herein.

As described herein, legumain is expressed in monocytes. Monocytes and macrophage originate from multipotential progenitor cells in bone marrow and play a pivotal role in host defense to pathogens, wound healing, angiogenesis, and various types of chronic inflammatory responses. Under chemokine and other cytokine induction monocytes migrate to tissues and differentiate into macrophages. Macrophages in various tissue and disease states vary in their morphology and function and have been given different names, e.g. Kupffer cells in liver, microglial cells in the central nervous system, and foam cells in atherosclerotic lesions. GM-CSF and M-CSF independently induce proliferation and differentiation of monocytes into distinct subsets of macrophages. Legumain is not detectable in freshly isolated unstimulated monocytes, but is up-regulated by both GM-CSF and M-CSF. Hashimoto, S., et al., *Serial analysis of gene expression in human monocytes and macrophages.* Blood, 1999. **94**(3): p. 837-44. M-CSF induced macrophages express a greater amount of legumain than GM-CSF induced macrophages.

According to the invention, legumain can influence monocyte/macrophage migration, infiltration, and antigen processing. M-CSF is a potent chemoattractant for cells of monocytes and macrophage lineages. Wang, J.M., et al., *Induction of monocyte migration by recombinant macrophage colony-stimulating factor.* J Immunol, 1988. **141**(2): p. 575-9. GM-CSF lacks chemotactic and chemokinetic effects, but enhances monocyte transendothelial migration in response to C5a or monocyte chemoattractant protein-1. Shang,

X.Z. and A.C. Issekutz, *Enhancement of monocyte transendothelial migration by granulocyte-macrophage colony-stimulating factor: requirement for chemoattractant and CD11a/CD18 mechanisms.* Eur J Immunol, 1999. **29**(11): p. 3571-82. Hence, legumain expression may be involved in increasing or modulating the migratory and infiltration activities of monocytes and/or macrophages.

The invention provides a method for modulating the migration and/or infiltration of cells that includes contacting the cells with legumain or an inhibitor of legumain. In general, increased levels of legumain can stimulate cellular migration and/or infiltration whereas inhibition of legumain can decrease cellular migration and/or infiltration. Such methods may be used *in vitro* or *in vivo*. Such methods may be useful not only for treating and preventing cancer but for treating and preventing inflammatory diseases, autoimmune diseases and atherosclerosis.

Imaging and Detection

In one embodiment, the agents and methods provided herein can be used to diagnose the location, extent, and pathologic composition of cancer anywhere within the body of a mammal. For example, detection of an agent capable of binding to or becoming activated by legumain can provide information regarding the location, shape, extent and pattern of the cancer. A reporter molecule, label or signaling compound can be attached to agents and inhibitors that can bind to, or be activated by, legumain. Such conjugates can then be used *in vivo* or *in vitro* to image, locate or otherwise detect the tissue to which the agent binds.

The reporter molecule, label or signaling compound that is linked to the agent or inhibitor will, of course, depend on the ultimate application of the invention. Where the aim is to provide an image of the tumor, one of skill in the art may desire to use a diagnostic agent that is detectable upon imaging, such as a paramagnetic, radioactive or fluorogenic agent. Such agents are available in the art, for example, as described and disclosed in U.S. Pat. 6,051,230 which is incorporated by reference herein in its entirety. Many diagnostic agents are known in the art to be useful for imaging purposes, as are methods for their

attachment to peptides and antibodies (see, e.g., U.S. Pat. Nos. 5,021,236 and 4,472,509, both incorporated herein by reference).

In the case of paramagnetic ions, one of skill may choose to use, for example, ions such as chromium (III), manganese (II), iron (III), iron (II), cobalt (II), nickel (II), copper (II), neodymium (III), samarium (III), ytterbium (III), gadolinium (III), vanadium (II), terbium (III), dysprosium (III), holmium (III) and erbium (III), with gadolinium being preferred. Ions useful in other contexts, such as X-ray imaging, include but are not limited to lanthanum (III), gold (III), lead (II), and especially bismuth (III). Moreover, in the case of radioactive isotopes for therapeutic and/or diagnostic application, one might mention iodine^{131}, iodine^{123}, iodine^{125}, technicium^{99}, indium^{111}, phosphorus^{32}, rhenium^{188}, rhenium^{186}, gallium^{67}, sulfur^{35}, copper^{67}, yttrium^{90}, tritium^{3} or astatine^{211}.

In some embodiments, agents and inhibitors may be conjugated with a dye or fluorescent moiety or intermediate such as biotin. Such conjugates can, for example, be used with infrared spectroscopy to detect and locate the tissues to which the agents bind.

In general, an assay for identifying legumain involves incubating a test sample under conditions that permit binding of legumain to a diagnostic agent, and measuring whether such binding has occurred. In some embodiments, the extent of binding between the diagnostic agent and legumain may be detected. Such information may be used to detect and assess the extent, spread or size of a cancerous tumor. A reporter molecule can be attached to any molecule that stably binds to legumain and that can be detected. For example, the reporter molecule can be attached to a legumain inhibitor or an anti-legumain antibody that is labeled as described above with paramagnetic ions, ions, radioactive isotopes, fluorescent dyes (e.g., fluorescein, rhodamine), enzymes and the like. It is understood that the choice of a reporter molecule will depend upon the detection system used.

Legumain Inhibitors

The present invention also provides a method of inhibiting cancer cell growth or tumor progression or tumor metastasis or invasion, for example, by inhibiting the expression or enzymatic activity of legumain. According to the

invention, legumain may be inhibited by any available mechanism, including by use of a legumain inhibitor, a cysteine protease inhibitor or by inhibition of legumain transcription or translation. In another embodiment, a legumain inhibitor may be used to deliver a drug to a legumain-expressing cell. When a legumain inhibitor is used to deliver a drug to a legumain-expressing cell, the legumain inhibitor preferably does not substantially block or inhibit the activity of the drug.

In some embodiments, the invention provides antagonists or agonists for legumain. Such antagonists or agonists may be inhibitors or co-factors of legumain, including proteins, peptides, carbohydrates, lipids or small molecular weight molecules, which interact with legumain to regulate or modulate its activity. Other molecules contemplated as agents for modulating legumain include antibodies targeted against legumain as well as molecules, compounds or peptides that mimic legumain substrates or inhibitors in structure and that bind to and form inactive complexes with legumain. Potential polypeptide antagonists include antibodies that react with legumain.

Legumain and other cysteine protease inhibitors are available in the art. See, e.g. Asgian, J.L., et al., *Aza-peptide epoxides: a new class of inhibitors selective for clan CD cysteine proteases.* J Med Chem, 2002. **45**(23): p. 4958-60; Niestroj, A.J., et al., *Inhibition of mammalian legumain by michael acceptors and AzaAsn-halomethylketones.* Biol Chem, 2002. **383**(7-8): p. 1205-14; and U.S. Patent 6,004,933, which are incorporated herein by reference. The invention contemplates using any such inhibitors as blocking or delivery agent in legumain-expressing cells.

In some embodiments, the legumain inhibitor is an inhibitor including formula formula **III**, **IV** or **V**:

$$\text{Hyd-(Xaa4)}_n\text{-Asn-CHO} \qquad \textbf{III}$$

$$\text{Xaa4-Asn-Y} \qquad \textbf{IV}$$

$$\text{Xaa4-azaAsn-Y} \qquad \textbf{V}$$

wherein:

Hyd is a hydrophilic group;

n is an integer of about 2 to about 5;

Xaa4 is an amino acid or an amino acid mimetic;

Y is alkyl, alkenyl, an epoxide or a Michael acceptor, wherein each alkyl, alkenyl, epoxide or Michael acceptor is optionally substituted with 1-3 halo or hydroxy, alkylamino, dialkylamino, alkyldialkylamino, or cycloalkyl, alkylcycloalkyl, alkenylcycloalkyl, aryl; (C_5 -C_{12})arylalkyl or (C_5 -C_{12})arylalkenyl,

wherein the aryl groups of the arylalky or arylalkenyl can be 0-4 heteroatoms selected from N, O and S, and are optionally substituted with halo, cyano, nitro, haloalkyl, amino, aminoalkyl, dialkylamino, alkyl, alkenyl, alkynyl, alkoxy, haloalkoxy, carboxyl, carboalkoxy, alkylcarboxamide, (C_5 -C_6)aryl, --O-(C_5 -C_6)aryl, arylcarboxamide, alkylthio or haloalkylthio; and

wherein the inhibitor is capable of binding to legumain.

Michael acceptors include functional groups that bond covalently to active-site nucleophiles. Such Michael acceptors can form the basis for potent and selective asparaginyl endopeptidase inhibitors. Examples of Michael acceptors useful in the inhibitors of the invention include N-Ac-L-Phe-$NHCH_2CH$ = CH-E and R-$NHCH_2CH$ = $CHCOOCH_3$ where E is an electron withdrawing group and R is an amino acid (e.g. Asn or azaAsn). Examples of suitable E electron withdrawing groups include CO_2CH_3, SO_2CH_3, CO_2H, CN, $CONH_2$ and C_6H_4-p-NO_2.

Legumain inhibitors that can be used include legumain catalytic inhibitors, such as cystatin, stefin, Tissue Inhibitor of Metalloproteinase 2 (TIMP-2) and a peptide having the sequence Ala-Leu-β-Asn-Ala-Ala (SEQ ID NO:15).

The Ala-Leu-β-Asn-Ala-Ala (SEQ ID NO:15) peptide is a synthetic suicide inhibitor that is useful as a legumain inhibitor, where the beta linkage is present to prevent hydrolysis. The SEQ ID NO:15 peptide will then remain bound to the legubicin catalytic site and block its activity.

The cystatins constitute a superfamily of evolutionarily related proteins that are all composed of at least one 100-120-residue domain with conserved sequence motifs. The single-domain human members of this superfamily are of

two major types. The type 1 cystatins (or stefins) A and B contain approximately 100 amino acid residues, lack disulfide bridges, and are synthesized without signal peptides. Cystatins of type 2 are secreted proteins of approximately 120 amino acid residues (M_r 13,000-14,000) and contain at least two characteristic intrachain disulfide bonds. The type 2 cystatins include the human cystatins C, D, S, SN, and SA, which are all products of genes located in the cystatin multigene locus on chromosome 20. Two recently identified type 2 cystatins, cystatin E/M and cystatin F (also called leukocystatin), are also secreted low molecular weight proteins but are more atypical in that they are glycoproteins and show only 30-35% sequence identity in alignments with the classical type 2 cystatins. They are, however, still functional inhibitors of family C1 cysteine peptidases. It has been shown that the cystatin inhibition of cysteine peptidases of the papain family is due to a tripartite wedge-shaped structure with very good complementarity to the active site clefts of such enzymes.

The three parts of the cystatin polypeptide chain included in the enzyme-binding domain are the N-terminal segment, a central loop-forming segment with the motif Gln-Xaa-Val-Xaa-Gly, and a second C-terminal hairpin loop typically containing a Pro-Trp pair.

Both types of cystatins inhibit legumain (cystatin C, E/M, F). Sequence alignments and molecular modeling have indicated that a loop located on the opposite side to the papain-binding surface, between the α-helix and the first strand of the main β-pleated sheet of the cystatin structure, may be involved in legumain binding.

Human cystatin C can inhibit legumain, as shown for example by Alvarez-Fernandez et al., J. Biol. Chem. 274: 19195-203 (1999). Several sequences for cystatin C are available, for example, in the ncbi database database. See website at ncbi.nlm.nih.gov. One such human cystatin C sequences has accession number CAA29096 (gi: 755738), and is provided below for easy reference (SEQ ID NO:17).

```
  1 MAGPLRAPLL LLAILAVALA VSPAAGSSPG KPPRLVGGPM
 41 DASVEEEGVR RALDFAVGEY NKASNDMYHS RALQVVRARK
 81 QIVAGVNYFL DVELGRTTCT KTQPNLDNCP FHDQPHLKRK
121 AFCSFQIYAV PWQGTMTLSK STCQDA
```

The fist 26 amino acids of this cystatin C polypeptide is a signal peptide (MAGPLRAPLL LLAILAVALA VSPAAG, SEQ ID NO:18) which when cleaved yields a mature cystatin C polypeptide of the following sequence (SEQ ID NO:19).

```
  27                                  SSPG KPPRLVGGPM
  41 DASVEEEGVR RALDFAVGEY NKASNDMYHS RALQVVRARK
  81 QIVAGVNYFL DVELGRTTCT KTQPNLDNCP FHDQPHLKRK
 121 AFCSFQIYAV PWQGTMTLSK STCQDA
```

As used herein, a mutated cystatin is a cystatin C polypeptide that lacks the N-terminal 10 ten amino acids or contains alanine at the Arg-8 position (R8A mutant), or contains alanine at the Leu-9 position (L9A mutant), or contains alanine at the Val-10 position (V10A) of the mature cystatin C polypeptide. In some embodiments, the mutated cystatin has all three mutations (R8A and L9A and V10A). Other mutations can also be present within the legumain binding region, but not in the region involved in cathepsin binding. These cystatin mutants will inhibit legumain specifically without inhibitory activity against cathepsin B or other Cathepsins.

Stefin B, also called cystatin B, can also inhibit cysteine proteinases such as legumain. Several sequences for cystatin B (stefin B) are available, for example, in the ncbi database database. See website at ncbi.nlm.nih.gov. One such human cystatin B sequences has accession number NP000091 (gi: 4503117), and is provided below for easy reference (SEQ ID NO:20).

```
  1 MMCGAPSATQ PATAETQHIA DQVRSQLEEK ENKKFPVFKA
 41 VSFKSQVVAG TNYFIKVHVG DEDFVHLRVF QSLPHENKPL
 81 TLSNYQTNKA KHDELTYF
```

In other embodiments, the invention provides anti-sense RNA or DNA molecules to modulate legumain expression, legumain translation and/or the degradation of legumain transcripts. For example, an anti-sense RNA or DNA that can hybridize to a legumain nucleic acid can be used as an anti-sense RNA or DNA for diminishing the expression of legumain. The legumain nucleic acid can have SEQ ID NO:2 or can have a sequence related to a genomic nucleotide sequence for human legumain that can be found in the NCBI database at accession number NT 026437 (gi: 29736559). See website at ncbi.nlm.nih.gov.

The degradation of legumain mRNA may also be increased upon exposure to small duplexes of synthetic double-stranded RNA through the use of RNA interference (siRNA or RNAi) technology. Scherr, M et al. Curr Med Chem 2003 10:245; Martinez, LA et al. 2002 PNAS 99: 14849. A process is therefore provided for inhibiting expression of legumain in a cell. The process includes introduction of RNA with partial or fully double-stranded character into the cell or into the extracellular environment. Inhibition is specific to legumain RNA because a nucleotide sequence from a portion of the legumain gene is chosen to produce inhibitory RNA. This process is effective in producing inhibition of gene expression.

The pSuppressorNeo vector for expressing hairpin siRNA, commercially available from IMGENEX (San Diego, California), can be used to generate siRNA for inhibiting legumain expression. The most critical part of the construction of the siRNA expression plasmid is the selection of the target region of the mRNA, which is currently a trial-and-error process. However, Elbashir et al. have provided guidelines that appear to work ~80% of the time. Elbashir, S.M., et al., *Analysis of gene function in somatic mammalian cells using small interfering RNAs.* Methods, 2002. **26**(2): p. 199-213. Accordingly for synthesis of synthetic siRNA, a target region may be selected preferably 50 to 100 nucleotides downstream of the start codon. The 5' and 3' untranslated regions and regions close to the start codon should be avoided as these may be richer in regulatory protein binding sites. The ideal sequence for a synthetic siRNA is 5'-AA(N19)UU, where N is any nucleotide in the mRNA sequence and should be approximately 50% G-C content. The selected sequence(s) can be compared to others in the human genome database to minimize homology to other known coding sequences (Blast search, for example, through the NCBI website).

However, for designing oligonucleotides for the expression vector, AA and UU dimers in the sequence are not needed. For the expression vector, siRNA can be designed to produce hairpin RNAs, in which both strands of an siRNA duplex would be included within a single RNA molecule. The individual motif can be 19-21 nucleotides long and correspond to the coding region of the legumain gene. However, Paddison and Hannon, 2002 have suggested use of

18-28 nucleotides. Paddison, P.J. and G.J. Hannon, *RNA interference: the new somatic cell genetics?* Cancer Cell, 2002. **2**(1): p. 17-23; Paddison, P.J., et al., *Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells.* Genes Dev, 2002. **16**(8): p. 948-58. The two motifs that form the inverted repeat are separated by a spacer of 4-9 nucleotides to permit formation of a hairpin loop. The transcriptional termination signal for 5 T's is added at the 3' end of the inverted repeat.

The siRNA insert can be prepared by synthesizing and annealing of two complementary oligonucleotides, and directly ligated this insert into the vector DNA. The resultant legumain suppressing vector DNA can be used to generate cell line that stably incorporates this vector and selection for retention of the construct can be achieved by selection of a linked marker. Such cell line is useful for preparing siRNA molecules for use in inhibiting legumain.

Mixtures and combinations of such siRNA molecules are also contemplated by the invention. These compositions can be used in the methods of the invention, for example, for treating or preventing cancer or metastasis. These compositions are also useful for modulating (e.g. decreasing) legumain expression.

The siRNA provided herein can selectively hybridize to RNA *in vivo* or *in vitro*. A nucleic acid sequence is considered to be "selectively hybridizable" to a reference nucleic acid sequence if the two sequences specifically hybridize to one another under physiological conditions or under moderate stringency hybridization and wash conditions. In some embodiments the siRNA is selectively hybridizable to an RNA (e.g. a legumain RNA) under physiological conditions. Hybridization under physiological conditions can be measured as a practical matter by observing interference with the function of the RNA. Alternatively, hybridization under physiological conditions can be detected *in vitro* by testing for siRNA hybridization using the temperature (e.g. 37 °C) and salt conditions that exist *in vivo*.

Moreover, as an initial matter, other in vitro hybridization conditions can be utilized to characterize siRNA interactions. Exemplary *in vitro* conditions include hybridization conducted as described in the Bio-Rad Labs ZetaProbe manual (Bio-Rad Labs, Hercules, Calif.); Sambrook et al., Molecular Cloning:

A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, (1989), or Sambrook et al., Molecular Cloning: A Laboratory Manual, 3rd ed., Cold Spring Harbor Laboratory Press, (2001)), expressly incorporated by reference herein. For example, hybridization can be conducted in 1 mM EDTA, 0.25 M $Na_2 HPO_4$ and 7% SDS at 42 °C, followed by washing at 42 °C in 1 mM EDTA, 40 mM $NaPO_4$, 5% SDS, and 1 mM EDTA, 40 mM $NaPO_4$, 1% SDS. Hybridization can also be conducted in 1 mM EDTA, 0.25 M $Na_2 HPO_4$ and 7% SDS at 60 °C, followed by washing in 1 mM EDTA, 40 mM $NaPO_4$, 5% SDS, and 1 mM EDTA, 40 mM $NaPO_4$, 1% SDS. Washing can also be conducted at other temperatures, including temperatures ranging from 37 °C to at 65 °C, from 42 °C to at 65 °C, from 37 °C to at 60 °C, from 50 °C to at 65 °C, from 37 °C to at 55 °C, and other such temperatures.

The siRNA employed in the compositions and methods of the invention may be synthesized either *in vivo* or *in vitro*. In some embodiments, the siRNA molecules are synthesized *in vitro* using methods, reagents and synthesizer equipment available to one of skill in the art. Endogenous RNA polymerases within a cell may mediate transcription *in vivo*, or cloned RNA polymerase can be used for transcription *in vivo* or *in vitro*. For transcription from a transgene or an expression construct *in vivo*, a regulatory region may be used to transcribe the siRNA strands.

Depending on the particular sequence utilized and the dose of double stranded siRNA material delivered, the compositions and methods may provide partial or complete loss of function for the target gene (legumain). A reduction or loss of gene expression in at least 99% of targeted cells has been shown for other genes. See, e.g., U.S. Patent 6,506,559. Lower doses of injected material and longer times after administration of the selected siRNA may result in inhibition in a smaller fraction of cells.

The siRNA may comprise one or more strands of polymerized ribonucleotide; it may include modifications to either the phosphate-sugar backbone or the nucleoside. The double-stranded siRNA structure may be formed by a single self-complementary RNA strand or two complementary RNA strands. siRNA duplex formation may be initiated either inside or outside the cell. The siRNA may be introduced in an amount that allows delivery of at least

one copy per cell. Higher doses of double-stranded material may yield more effective inhibition.

Inhibition is sequence-specific in that nucleotide sequences corresponding to the duplex region of the RNA are targeted for genetic inhibition. siRNA containing nucleotide sequences identical to a portion of the target gene is preferred for inhibition. However, siRNA sequences with insertions, deletions, and single point mutations relative to the target sequence have also been found to be effective for inhibition. Thus, sequence identity may optimized by alignment algorithms known in the art and calculating the percent difference between the nucleotide sequences. Alternatively, the duplex region of the RNA may be defined functionally as a nucleotide sequence that is capable of hybridizing with a portion of the target gene transcript.

The siRNA may be directly introduced into the cell (i.e., intracellularly); or introduced extracellularly into a cavity, interstitial space, into the circulation of an organism, introduced orally, or may be introduced by bathing an organism in a solution containing siRNA. Methods for oral introduction include direct mixing of siRNA with food of the organism, as well as engineered approaches in which a species that is used as food is engineered to express an siRNA, then fed to the organism to be affected. Physical methods of introducing nucleic acids include injection directly into the cell or extracellular injection into the organism of an siRNA solution.

The siRNA may also be delivered in vitro to cultured cells using transfection agents available in the art such as lipofectamine or by employing viral delivery vectors such as those from lentiviruses. Such *in vitro* delivery can be performed for testing purposes or for therapeutic purposes. For example, cells from a patient can be treated *in vitro* and then re-administered to the patient.

The advantages of using siRNA include: the ease of introducing double-stranded siRNA into cells, the low concentration of siRNA that can be used, the stability of double-stranded siRNA, and the effectiveness of the inhibition. The ability to use a low concentration of a naturally-occurring nucleic acid avoids several disadvantages of anti-sense interference.

Anti-Legumain Antibodies

The invention provides antibody preparations directed against legumain, for example, antibodies capable of binding a polypeptide having SEQ ID NO:1. For example, in some embodiments, the antibody can bind a legumain epitope that includes the following sequence CGMKRASSPVPLPP (SEQ ID NO:16). This sequence (SEQ ID NO:16) is near the protease cleavage site that activates legumain. Therefore, this antibody preferencially recognizes activated legumain compared to legumain proenzyme. Such anbodies are desirable to detect activated legumain proteases, which are associated with pathologiical cancer and tumor cells. Moreover, antibody preparations of the invention can serve as inhibitors of legumain activity and therefore act as therapeutic agents.

Methods are provided to prepare and screen for antibodies that preferentially recognize activated proteases, for example, asparaginyl endopeptidases. A peptide sequence (e.g. SEQ ID NO:16) that is near the proteolytic leavage site is used as antigen to raise polyclonal or monoclonal antibodies. The resultant antibodies are selected for binding to the selected peptide sequence, for binding to the activated protease and then for inhibition of the activated protease. The antibody recognition site at the free end of the peptide is only be available in activated proteases, hence desirable antibodies bind to the activated protease and exhibit substantially less binding to the non-activated protease. Inhibitory antibodies are selected by screening the antibodies for inhibition of proteolysis during activity assays.

Antibody molecules belong to a family of plasma proteins called immunoglobulins, whose basic building block, the immunoglobulin fold or domain, is used in various forms in many molecules of the immune system and other biological recognition systems. A typical immunoglobulin has four polypeptide chains, containing an antigen binding region known as a variable region and a non-varying region known as the constant region.

Native antibodies and immunoglobulins are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies between the heavy chains of different immunoglobulin isotypes. Each

heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (VH) followed by a number of constant domains. Each light chain has a variable domain at one end (VL) and a constant domain at its other end. The constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light and heavy chain variable domains (Clothia et al., J. Mol. Biol. 186, 651-66, 1985); Novotny and Haber, Proc. Natl. Acad. Sci. USA 82, 4592-4596 (1985).

Depending on the amino acid sequences of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are at least five (5) major classes of immunoglobulins: IgA, IgD, IgE, IgG and IgM, and several of these may be further divided into subclasses (isotypes), e.g. IgG-1, IgG-2, IgG-3 and IgG-4; IgA-1 and IgA-2. The heavy chains constant domains that correspond to the different classes of immunoglobulins are called alpha (α), delta (δ), epsilon (ε), gamma (γ) and mu (μ), respectively. The light chains of antibodies can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino sequences of their constant domain. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

The term "variable" in the context of variable domain of antibodies, refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies. The variable domains are for binding and determine the specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed through the variable domains of antibodies. It is concentrated in three segments called complementarity determining regions (CDRs) also known as hypervariable regions both in the light chain and the heavy chain variable domains.

The more highly conserved portions of variable domains are called the framework (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a β-sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the β-sheet structure. The CDRs in each chain are held together in close

proximity by the FR regions and, with the CDRs from the other chain, contribute to the formation of the antigen-binding site of antibodies. The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

An antibody that is contemplated for use in the present invention thus can be in any of a variety of forms, including a whole immunoglobulin, an antibody fragment such as Fv, Fab, and similar fragments, a single chain antibody which includes the variable domain complementarity determining regions (CDR), and the like forms, all of which fall under the broad term "antibody", as used herein. The present invention contemplates the use of any specificity of an antibody, polyclonal or monoclonal, and is not limited to antibodies that recognize and immunoreact with a specific antigen. In preferred embodiments, in the context of both the therapeutic and screening methods described below, an antibody or fragment thereof is used that is immunospecific for an antigen or epitope of the invention.

The term "antibody fragment" refers to a portion of a full-length antibody, generally the antigen binding or variable region. Examples of antibody fragments include Fab, Fab', $F(ab')_2$ and Fv fragments. Papain digestion of antibodies produces two identical antigen binding fragments, called the Fab fragment, each with a single antigen binding site, and a residual "Fc" fragment, so-called for its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen binding fragments that are capable of cross-linking antigen, and a residual other fragment (which is termed pFc'). Additional fragments can include diabodies, linear antibodies, single-chain antibody molecules, and multispecific antibodies formed from antibody fragments. As used herein, "functional fragment" with respect to antibodies, refers to Fv, F(ab) and $F(ab')_2$ fragments.

Antibody fragments contemplated by the invention are therefore not full-length antibodies but do have similar or improved immunological properties relative to an anti-legumain antibody. Such antibody fragments may be as small as about 4 amino acids, 5 amino acids, 6 amino acids, 7 amino acids, 9 amino acids, about 12 amino acids, about 15 amino acids, about 17 amino acids, about

18 amino acids, about 20 amino acids, about 25 amino acids, about 30 amino acids or more. In general, an antibody fragment of the invention can have any upper size limit so long as it binds with specificity to legumain, e.g. a polypeptide having SEQ ID NO:1.

Antibody fragments retain some ability to selectively bind with its antigen. Some types of antibody fragments are defined as follows:

(1) Fab is the fragment that contains a monovalent antigen-binding fragment of an antibody molecule. A Fab fragment can be produced by digestion of whole antibody with the enzyme papain to yield an intact light chain and a portion of one heavy chain.

(2) Fab' is the fragment of an antibody molecule can be obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain. Two Fab' fragments are obtained per antibody molecule. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxyl terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region.

(3) $(Fab')_2$ is the fragment of an antibody that can be obtained by treating whole antibody with the enzyme pepsin without subsequent reduction. $F(ab')_2$ is a dimer of two Fab' fragments held together by two disulfide bonds.

(4) Fv is the minimum antibody fragment that contains a complete antigen recognition and binding site. This region consists of a dimer of one heavy and one light chain variable domain in a tight, non-covalent association (V_H -V_L dimer). It is in this configuration that the three CDRs of each variable domain interact to define an antigen binding site on the surface of the V_H -V_L dimer. Collectively, the six CDRs confer antigen binding specificity to the antibody. However, even a single variable domain (or half of an Fv including only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

(5) Single chain antibody ("SCA"), defined as a genetically engineered molecule containing the variable region of the light chain, the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule. Such single chain antibodies are also referred to as "single-chain Fv" or "sFv" antibody fragments. Generally, the Fv

polypeptide further includes a polypeptide linker between the VH and VL domains that enables the sFv to form the desired structure for antigen binding. For a review of sFv see Pluckthun in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenburg and Moore eds. Springer-Verlag, N.Y., pp. 269-315 (1994).

The term "diabodies" refers to a small antibody fragments with two antigen-binding sites, which fragments comprise a heavy chain variable domain (VH) connected to a light chain variable domain (VL) in the same polypeptide chain (VH-VL). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161, and Hollinger et al., Proc. Natl. Acad Sci. USA 90: 6444-6448 (1993).

Methods for preparing polyclonal antibodies are available to those skilled in the art. See, for example, Green, et al., Production of Polyclonal Antisera, in: Immunochemical Protocols (Manson, ed.), pages 1-5 (Humana Press); Coligan, et al., Production of Polyclonal Antisera in Rabbits, Rats Mice and Hamsters, in: Current Protocols in Immunology, section 2.4.1 (1992), which are hereby incorporated by reference.

Methods for preparing monoclonal antibodies are likewise available to one of skill in the art. See, for example, Kohler & Milstein, Nature, 256:495 (1975); Coligan, et al., sections 2.5.1-2.6.7; and Harlow, et al., in: Antibodies: A Laboratory Manual, page 726 (Cold Spring Harbor Pub. (1988)), which are hereby incorporated by reference. Monoclonal antibodies can be isolated and purified from hybridoma cultures by a variety of well-established techniques. Such isolation techniques include affinity chromatography with Protein-A Sepharose, size-exclusion chromatography, and ion-exchange chromatography. See, e.g., Coligan, et al., sections 2.7.1-2.7.12 and sections 2.9.1-2.9.3; Barnes, et al., Purification of Immunoglobulin G (IgG), in: Methods in Molecular Biology, Vol. 10, pages 79-104 (Humana Press (1992).

Methods of *in vitro* and *in vivo* manipulation of monoclonal antibodies are also available to those skilled in the art. For example, monoclonal antibodies to be used in accordance with the present invention may be made by the

hybridoma method first described by Kohler and Milstein, Nature 256, 495 (1975), or may be made by recombinant methods, e.g., as described in U.S. Pat. No. 4,816,567. The monoclonal antibodies for use with the present invention may also be isolated from phage antibody libraries using the techniques described in Clackson et al. Nature 352: 624-628 (1991), as well as in Marks et al., J. Mol Biol. 222: 581-597 (1991). Another method involves humanizing a monoclonal antibody by recombinant means to generate antibodies containing human specific and recognizable sequences. See, for review, Holmes, et al., J. Immunol., 158:2192-2201 (1997) and Vaswani, et al., Annals Allergy, Asthma & Immunol., 81:105-115 (1998).

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional polyclonal antibody preparations that typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In additional to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates that the antibody preparation is a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method.

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567); Morrison et al. Proc. Natl. Acad Sci. 81, 6851-6855 (1984).

Methods of making antibody fragments are also known in the art (see for example, Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York, (1988), incorporated herein by reference). Antibody fragments of the present invention can be prepared by proteolytic hydrolysis of the antibody or by expression in *E. coli* of DNA encoding the fragment. Antibody fragments can be obtained by pepsin or papain digestion of whole antibodies conventional methods. For example, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted $F(ab')_2$. This fragment can be further cleaved using a thiol reducing agent, and optionally a blocking group for the sulfhydryl groups resulting from cleavage of disulfide linkages, to produce 3.5S Fab' monovalent fragments. Alternatively, an enzymatic cleavage using pepsin produces two monovalent Fab' fragments and an Fc fragment directly. These methods are described, for example, in U.S. Patents No. 4,036,945 and No. 4,331,647, and references contained therein. These patents are hereby incorporated in their entireties by reference.

Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical, or genetic techniques may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody. For example, Fv fragments comprise an association of V_H and V_L chains. This association may be non-covalent or the variable chains can be linked by an intermolecular disulfide bond or cross-linked by chemicals such as glutaraldehyde. Preferably, the Fv fragments comprise V_H and V_L chains connected by a peptide linker. These single-chain antigen binding proteins (sFv) are prepared by constructing a structural gene comprising DNA sequences encoding the V_H and V_L domains connected by an oligonucleotide. The structural gene is inserted into an expression vector, which is subsequently introduced into a host cell such as *E. coli*. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing sFvs are described, for example, by Whitlow, et al., Methods: a Companion to Methods in Enzymology, Vol. 2, page 97 (1991);

Bird, et al., Science 242:423-426 (1988); Ladner, et al, US Patent No. 4,946,778; and Pack, et al., Bio/Technology 11:1271-77 (1993).

Another form of an antibody fragment is a peptide coding for a single complementarity-determining region (CDR). CDR peptides ("minimal recognition units") are often involved in antigen recognition and binding. CDR peptides can be obtained by cloning or constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region from RNA of antibody-producing cells. See, for example, Larrick, et al., Methods: a Companion to Methods in Enzymology, Vol. 2, page 106 (1991).

The invention contemplates human and humanized forms of non-human (e.g. murine) antibodies. Such humanized antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a nonhuman species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity.

In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, humanized antibodies will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see: Jones et al., Nature 321, 522-525 (1986); Reichmann et al., Nature 332, 323-329 (1988); Presta, Curr. Op. Struct. Biol. 2, 593-596 (1992); Holmes, et al., J. Immunol., 158:2192-2201

(1997) and Vaswani, et al., Annals Allergy, Asthma & Immunol., 81:105-115 (1998).

The invention also provides methods of mutating antibodies to optimize their affinity, selectivity, binding strength or other desirable property. A mutant antibody refers to an amino acid sequence variant of an antibody. In general, one or more of the amino acid residues in the mutant antibody is different from what is present in the reference antibody. Such mutant antibodies necessarily have less than 100% sequence identity or similarity with the reference amino acid sequence. In general, mutant antibodies have at least 75% amino acid sequence identity or similarity with the amino acid sequence of either the heavy or light chain variable domain of the reference antibody. Preferably, mutant antibodies have at least 80%, more preferably at least 85%, even more preferably at least 90%, and most preferably at least 95% amino acid sequence identity or similarity with the amino acid sequence of either the heavy or light chain variable domain of the reference antibody. One method of mutating antibodies involves affinity maturation using phage display.

The invention is therefore directed to a method for selecting antibodies and/or antibody fragments or antibody polypeptides with desirable properties. Such desirable properties can include increased binding affinity or selectivity for the epitopes of the invention

The antibodies and antibody fragments of the invention are isolated antibodies and antibody fragments. An isolated antibody is one that has been identified and separated and/or recovered from a component of the environment in which it was produced. Contaminant components of its production environment are materials that would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. The term "isolated antibody" also includes antibodies within recombinant cells because at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step

If desired, the antibodies of the invention can be purified by any available procedure. For example, the antibodies can be affinity purified by binding an antibody preparation to a solid support to which the antigen used to raise the

antibodies is bound. After washing off contaminants, the antibody can be eluted by known procedures. Those of skill in the art will know of various techniques common in the immunology arts for purification and/or concentration of polyclonal antibodies, as well as monoclonal antibodies (see for example, Coligan, et al., Unit 9, Current Protocols in Immunology, Wiley Interscience, 1991, incorporated by reference).

In some embodiments, the antibody will be purified as measurable by at least three different methods: 1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight; 2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequentator; or 3) to homogeneity by SDS-PAGE under reducing or non-reducing conditions using Coomasie blue or, preferably, silver stain.

Methods of Detecting and Isolating Agents that can Modulate Legumain

The invention further provides screening assays that are useful for generating or identifying therapeutic agents for prevention and treatment of cancer and assays for generating or identifying agents that modulate legumain activity. In particular, the legumain substrates, legumain inhibitors, legumain nucleic acids and legumain proteins identified herein may be used in a variety of assays for detecting legumain and for identifying factors that interact with legumain nucleic acids or with the legumain protein. For example, in some embodiments, assays can be performed to assess whether a potential prodrug can be activated by legumain cleavage.

Prodrug conversion assay may be carried out in a variety of ways. For example, a cytotoxic agent can be linked to a peptide that has a legumain cleavage site as described above. Cultured non-cancerous cells and cancerous cells (e.g., those that express legumain) may then be separately exposed to the prodrug. After a suitable exposure time, the cultures are observed to ascertain whether the cancerous cells are preferentially killed or inhibited in their growth. The culture fluids may also be assayed to determine whether the prodrug has been cleaved in a manner that is consistent with legumain cleavage. Before such assays are performed, prodrug candidates can be screened to ascertain whether

they are efficiently cleaved by legumain. In some embodiments, the cancer cells employed overexpress legumain.

Further assays can be performed to assess the *in vivo* toxicity and *in vivo* efficacy for treating cancer. Suitable animal models and tumor cell lines can be used for these purposes. For example, mice, rats or other model animals with a propensity for developing cancer can be employed. Alternatively, small tumors or tumor cells or cancer cells that overexpress legumain can be transplanted into normal animals. Some of the animals that received tumors, tumor cells or legumain+ cells are then treated with the prodrug. Other of those animals can be treated with the cytotoxic agent that forms part of the prodrug. Tumor growth and physical signs can be monitored daily including any gross evidence of tumor necrosis, local tumor ulceration as well as evidence of toxicity including mobility, response to stimulus, eating, and weight of each animal. Prodrugs that effectively reduce or eliminate tumors while having minimal negative effects on the health, lifespan and tissue integrity of the model animal are selected for development as a prodrug.

Assays may be used to identify agents that can interact with a cancer cell of interest. A wide variety of assays may be used for this purpose. See, for example, the assays carried out within the National Cancer Institute's "*In Vitro* Cell Line Screening Project." In general, such an assay can involve contacting a cancer cell of interest with at least one agent and observing whether the agent kills the cancer cell and/or has other deleterious effects upon that cell.

Methods available in the art can also be used for determining whether the agents of the invention interact with the membrane of a cancer cell of interest. For example, the agent can be labeled with a reporter molecule that permits detection of the agent. After labeling, the agents can be contacted with the cancer cell of interest for a time and under conditions that permit binding or association of the agent to cellular membranes. The cells can be washed with physiological solutions to remove unbound or unassociated agents, and the cells can then be observed to ascertain whether the reporter molecule is bound or associated with the cells or the cellular membranes. In another embodiment, one of skill in the art can test whether the agent(s) can penetrate the membranes of selected cancer cells. This may be done by examining whether the reporter

molecule remains associated with the cellular membranes of the cancer cell or whether the reporter molecule becomes associated with the interior of the cell.

Reporter molecules that can be employed include any detectable compound or molecule that is conjugated directly or indirectly to an agent of the invention. The label may itself be detectable (e.g., radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition that is detectable.

Deleterious effects upon the cancer cell of interest can also be detected as an indication of an interaction between an agent of the invention and the cell. Such deleterious effects can involve any evidence that the agent has had an adverse or cytotoxic effect upon the cell. For example, one of skill in the art can test whether the agent(s) kill the cancer cell, cause membrane depolarization, cause permeabilization of the membranes of the cell, or tend to lyse the cancer cells.

Pluralities of assays can be performed in parallel with different agents at different concentrations to obtain a differential response to the various concentrations. Typically, at least one control assay is included in the testing. Such a control can be a negative control involving exposure of the cancer cells of interest to a physiologic solution containing no agents. Another control can involve exposure of the cancer cell of interest to an agent that has already been observed to adversely affect the cancer cell of interest, or a second cell that is related to the cell of interest. Another control can involve exposing a cell of interest to a known therapeutic compound that has a desired affect on the cancer cell of interest, for example, an anti-cancer agent with known efficacy at a particular concentration or dosage. One of skill in the art can readily select control compounds and conditions that facilitate screening and analysis of the effects of the cyclic peptides on a cancer cell of interest.

Any cell type can be assayed by these methods. For example, any mammalian or other animal cancer cell type can be screened to assess whether the agents of the invention can selectively interact therewith. Mammalian or other animal cells can also be screened to ascertain whether the agents of the invention selectively interact therewith and/or to determine whether the agents of

the invention do not interact, bind, lyse, kill or otherwise adversely affect the viability of the mammalian or other animal cell.

Conditions for screening include conditions that are used by one of skill in the art to grow, maintain or otherwise culture cell types of interest. Cancer cell types of interest should be assayed under conditions where they would be healthy but for the presence of the agents. Controls can be performed where the cell types are maintained under the selected culture conditions and not exposed to an agent, to assess whether the culture conditions influenced the viability of the cells. One of skill in the art can also perform the assay on cells that have been washed in simple physiological solutions, such as buffered saline, to eliminate, or test for, any interaction between the agents or cells and the components in the culture media. However, culture conditions for the assays generally include providing the cells with the appropriate concentration of nutrients, physiological salts, buffers and other components typically used to culture or maintain cells of the selected type. A variety of other reagents may be included in the screening assay. These include reagents like salts, neutral proteins, albumin, and serum (e.g. fetal calf serum) that are used to mimic the physiologic state of the cell types of interest. Conditions and media for culturing, growing and maintaining cells are available to one of skill in the art.

The selected reagents and components are added to the assay in the order selected by one of skill in the art. In general, the agents are added last to start the assay. Assays are performed at any suitable temperature, typically between 4°C and 40°C. For example, the temperature may generally range from about room temperature (about 20°C) to about 37°C. Incubation periods are selected to ascertain the optimal range of activity, or to insure that the agents do not adversely affect the cell type of interest. However, incubation times can be optimized to facilitate rapid high-throughput screening. Typically, incubation times are between about one minute and about five days, such as a range from about 30 minutes to about 3 days.

Agents having the desired activity *in vitro* may be tested for activity and/or lack of toxicity *in vivo*, in an appropriate animal model. Such animal models include primates as well as mice, rats, rabbits, cats, dogs, pigs, goats, cattle or horses. For example, the mouse is a convenient animal model for

testing whether agents of the invention have toxic effects and/or to determine whether the agents can inhibit the growth of a cancer cell.

One of skill in the art can readily perform *in vivo* evaluation of the agents of the invention. For toxicity testing, a series of agents at different test dosages can be separately administered to different animals. A single dose or, a series of dosages can be administered to the animal. A test period is selected that permits assessment of the effects of the agent(s) on the animal. Such a test period can run from about one day to about several weeks or months.

The effect of a agent(s) on an animal can be determined by observing whether the agent adversely affects the behavior (e.g., lethargy, hyperactivity) and physiological state of the animal over the course of test period. The physiological state of the animal can be assessed by standard procedures. For example, during the test period one of skill in the art can draw blood and collect other bodily fluids to test, for example, for various enzymes, proteins, metabolites, and the like. One of skill in the art can also observe whether the animal has bloating, loss of appetite, diarrhea, vomiting, blood in the urine, loss of consciousness, and a variety of other physiological problems. After the test period, the animal can be sacrificed and anatomical, pathological, histological and other studies can be performed on the tissues or organs of the animal.

In general, to determine whether one or more agents of the invention can inhibit cancer cell growth, mice are infected with the selected cancer and a selected test dosage of one or more agents is administered shortly thereafter. Mice are observed over the course of several days to several weeks to ascertain whether the agents protect the mice from the cancer. At the end of the test period, mice can be sacrificed and examined to ascertain whether the agent has optimally protected the mice from cancer and/or to determine whether any adverse side effects have occurred.

Controls are used to establish the effects of the cancer when the agent is not administered. Other controls can also be performed, for example, to determine the safety and efficacy of the present agents compared to that of known anti-cancer compounds.

Binding assays between legumain and other agents may be carried out in several formats, including cell-based binding assays, solution-phase assays and

immunoassays. In general, test samples or compounds are incubated with legumain for a specified period of time followed by measurement of binding between legumain and the test sample or compound. A label or reporter molecule attached to the legumain, test sample or compound may be detected by use of microscopy, fluorimetry, a scintillation counter, or any available immunoassay. Binding can also be detected by labeling legumain in a competitive radioimmunoassay. Alternatively, legumain may be modified with an unlabeled epitope tag (e.g., biotin, peptides, His_6, FLAG, myc etc.) and bound to proteins such as streptavidin, anti-peptide or anti-protein antibodies that have a detectable label as described above. Additional forms of legumain containing epitope tags may be used in solution and immunoassays.

Methods for identifying compounds or molecules that interact with legumain are also encompassed by the invention. In general, an assay for identifying compounds or molecules that interact with legumain involves incubating legumain with a test sample that may contain such a compound or molecule under conditions that permit binding of the compound or molecule to legumain, and measuring whether binding has occurred. Legumain may be purified or present in mixtures, such as in cultured cells, tissue samples, body fluids or culture medium. Assays may be developed that are qualitative or quantitative. Quantitative assays can be used for determining the binding parameters (affinity constants and kinetics) of the compound or molecule for legumain and for quantifying levels of biologically active compounds and molecules in mixtures. Assays may also be used to evaluate the binding of a compound or molecule to fragments, analogs and derivatives of legumain and to identify new legumain family members.

The compound or molecule in a test sample may be substantially purified or present in a crude mixture. Binding compounds and molecules may be nucleic acids, proteins, peptides, carbohydrates, lipids or small molecular weight organic compounds. The compounds and molecules may be further characterized by their ability to increase or decrease legumain activity in order to determine whether they act as an agonist or an antagonist.

Legumain may be purified or be present in mixtures, such as in cultured cells, tissue samples, body fluids or culture medium. Assays may be developed

that are qualitative or quantitative, with the latter being useful for determining the conversion rate or the binding parameters (affinity constants and kinetics) of the agent in its interaction with legumain and for quantifying levels of legumain in mixtures. Assays may also be used to detect fragments, analogs and derivatives of legumain and to identify new legumain family members.

Legumain nucleic acids are also useful for identification of factors that interact with the legumain promoter and that modulate legumain expression. Such factors may be intracellular proteins such as DNA binding proteins that interact with regulatory sequences that control legumain transcription, for example, the legumain promoter. As an example, hybrid constructs may be used that include a nucleic acid encoding the legumain promoter fused to a nucleic acid encoding a marker protein. The legumain promoter can be found within the genomic nucleotide sequence for human legumain that is available in the NCBI database at accession number NT 026437 (gi: 29736559). *See* website at ncbi.nlm.nih.gov. The marker protein can be any marker protein available to one of skill in the art. For example, the marker protein can be luciferase, green fluorescence protein (GFP) or CAT.

Such hybrid constructs are used for in vitro or in vivo transcription assays to identify factors that modulate legumain expression. Factors that depress or diminish legumain expression are particularly useful. Expression or transcription levels can be assessed using any method available to one of skill in the art for measuring RNA levels. For example, RNA levels can be assessed by northern analysis, reverse transcriptase analysis, reverse transcriptase coupled with polymerase chain reaction (RT-PCR) analysis and other methods. Chemical libraries can be screened using such methods for small molecule compounds that block legumain transcription.

Compositions

The prodrugs and compounds of the invention as well as antibodies and inhibitors of legumain can be formulated as pharmaceutical compositions and administered to a mammalian host, such as a human patient in a variety of forms adapted to the chosen route of administration. Routes for administration include,

for example, oral, parenteral, intraperitoneal, intravenous and intraarterial routes.

Solutions of the agents or their salts can be prepared in water or saline, and optionally mixed with a nontoxic surfactant. Formulations for intravenous or intraarterial administration may include sterile aqueous solutions that may also contain buffers, liposomes, diluents and other suitable additives.

The pharmaceutical dosage forms suitable for injection or infusion can include sterile aqueous solutions or dispersions comprising the active ingredient that are adapted for administration by encapsulation in liposomes. In some embodiments, the liposomes contain a somewhat more hydrophobic prodrug or asparaginyl endopeptidase inhibitor. Thus, reduced numbers or no hydrophilic groups (e.g. no Hyd) may be present on the prodrug or asparaginyl endopeptidase inhibitor. Instead, a less hydrophilic protecting group (e.g. t-BOC) may be used instead of Hyd. Alternatively, no Hyd or protecting groups may be used. For example, in some embodiments, legubicin may be used in the liposome compositions instead of LEG-3. The ultimate dosage form should be sterile, fluid and stable under the conditions of manufacture and storage.

Sterile injectable solutions are prepared by incorporating the agents and inhibitors in the required amount in the appropriate solvent with various of the other ingredients, as required, followed by filter sterilization.

Useful dosages of the agents and inhibitors can be determined by comparing their *in vitro* activity, and *in vivo* activity in animal models. Methods for the extrapolation of effective dosages in mice, and other animals, to humans are known to the art; for example, see U.S. Pat. No. 4,938,949. The compound can be conveniently administered in unit dosage form.

The desired dose may conveniently be presented in a single dose or as divided doses administered at appropriate intervals, for example, as two, three, four or more sub-doses per day. The sub-dose itself may be further divided, for example, into a number of discrete loosely spaced administrations; such as multiple oral, intraperitoneal or intravenous doses. For example, it is desirable to administer the present compositions intravenously over an extended period, either by continuous infusion or in separate doses.

In some instances, the agents and inhibitors can be administered orally, in combination with a pharmaceutically acceptable vehicle such as an inert diluent or an assimilable edible carrier. They may be enclosed in hard or soft shell gelatin capsules, may be compressed into tablets, or may be incorporated directly with the food of the patient's diet. For oral therapeutic administration, they may be combined with one or more excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations may contain at least 0.1% of active compound. The percentage of the compositions and preparations may, of course, be varied. The amount of compound in such therapeutically useful compositions is such that an effective dosage level will be obtained.

The tablets, troches, pills, capsules, and the like may also contain the following: binders such as gum tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, fructose, lactose or aspartame or a flavoring agent such as peppermint, oil of wintergreen, or cherry flavoring may be added. When the unit dosage form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier, such as a vegetable oil or a polyethylene glycol. Various other materials may be present as coatings or to otherwise modify the physical form of the solid unit dosage form. For instance, tablets, pills, or capsules may be coated with gelatin, wax, shellac or sugar and the like. A syrup or elixir may contain the active compound, sucrose or fructose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavoring such as cherry or orange flavor. Of course, any material used in preparing any unit dosage form should be pharmaceutically acceptable and substantially non-toxic in the amounts employed. In addition, the active compound may be incorporated into sustained-release preparations and devices.

Useful solid carriers include finely divided solids such as talc, clay, microcrystalline cellulose, silica, alumina and the like. Useful liquid carriers include water, alcohols or glycols or water-alcohol/glycol blends, in which the present compounds can be dissolved or dispersed at effective levels, optionally with the aid of non-toxic surfactants. Adjuvants such as fragrances and

additional antimicrobial agents can be added to optimize the properties for a given use.

In some embodiments, the prodrugs or inhibitors are linked to polyethylene glycol (PEG). For example, one of skill in the art may choose to link a drug to PEG to form the following pegylated prodrug:

Drug – (linker) – Legumain cleavage site – (linker) – PEG

wherein the linker can be a covalent bond, an amino acid, a peptidyl sequence, a sugar residue, a glycol chain or an alkylene chain.

Thickeners such as synthetic polymers, fatty acids, fatty acid salts and esters, fatty alcohols, modified celluloses or modified mineral materials can also be employed with liquid carriers to form spreadable pastes, gels, ointments, soaps, and the like, for application directly to the skin of the user.

The therapeutically effective amount of prodrug, compound, or inhibitor necessarily varies with the subject and the disease or physiological problem to be treated. As one skilled in the art would recognize, the amount can be varied depending on the method of administration. The amount of the agent or inhibitor for use in treatment will vary not only with the route of administration, but also the nature of the condition being treated and the age and condition of the patient and will be ultimately at the discretion of the attendant physician or clinician.

The pharmaceutical compositions of the invention can include an effective amount of at least one of the agents of the invention, or two or more different agents of the invention. These compositions also include a pharmaceutically effective carrier.

The invention will be described with reference to the following non-limiting examples.

Example 1: Legumain is Expressed in Tumors

Example 1 demonstrates that legumain is over-expressed in human tumors.

Materials and Methods

Reagents and cell lines

Rabbit polyclonal antisera against human legumain, as well as 293 cells stably expressing human legumain, were kindly provided by Dr. D. Roodman (Department of Medicine and Hematology, University of Texas Health Science Center, San Antonio, TX). A legumain substrate peptide was synthesized by and purchased from Bachem (King of Prussia, PA). Doxorubicin was purchased from Sigma (St. Louis, MO). Costar migration chambers were obtained from Corning Incorporated (Corning, NY). Vitrogen was obtained from Cohesion Technologies (Palo Alto, CA). Mouse monoclonal antibody specific for human integrin β1 was obtained from Dr. R. Klemke (The Scripps Research Institute). The DMEM media was obtained from Invitrogen (Carlsbad, CA). The CT26 murine colon carcinoma cell line, the C1300 mouse neuroblastoma cell line anf human HT1080 fibrosarcoma cells were purchased from the American Type Culture Collection (ATCC). The 293 cells used to construct tetracycline-regulated cell lines expressing legumain were obtained from Stratagene (La Jolla, CA). Multiple tumor tissue arrays were provided by Cooperative Human Tissue Network, National Cancer Institute.

Rapid isolation of tumor endothelial cells and mRNA extraction

CD31 antibody coated Dynabeads were prepared by mixing 300 µl bead suspension with 500 µl PBSA (PBS, 1% BSA). Biotinylated anti mouse CD31 antibody (20 µg) was added to the suspension and association of antibody to beads was for 20 minutes at 4°C . The beads were washed 3 times with PBS to remove unbound antibody. CT26 tumors grown to ~1.5 cm greatest diameter were surgically removed and cooled to 4°C for following steps, and the tumor minced into 1 mm^3 bits with sterile scissors. The minced tumor was gently pressed through metal meshes and filtered through a 40 µm Falcon cell strainer (Becton Dickinson, Franklin Lakes, NJ) to rapidly recover the single cell suspensions. Streptavidin conjugated paramagnetic Dynabeads (Dynal, Lake Success, NY) coated with biotinylated anti-mouse CD31 antibody (Mec 13.3, PharMingen, La Jolla, CA) were immediately added to the single cell suspensions. Capture by beads of CD31 positive cells was conducted at 4°C for 20 minutes with gentle agitation. Beads with bound CD31 positive cells were recovered with a magnetic trap column and washed three times with cold phosphate buffered saline (PBS). Unbound CD31 negative cells were collected separately and were recovered by centrifugation at 1000 rpm for 3 minutes. Both CD31 positive and CD31 negative cells were used for mRNA extraction (Qiagene mRNA direct kit). The concentration of mRNA was quantified with RiboGreen RNA quantification reagents (Molecular Probes, Eugene, OR).

Differential gene expression profiling using restriction fragment differential display

Five hundred ng mRNA was used for differential profiling using the displayPROFILE method (Display Systems Biotech, Vista, CA). The mRNA was first used to synthesize double stranded cDNA. The resultant double stranded DNA was digested with Taq I and adaptors were ligated onto the fragment ends. Display primer was used to PCR amplify the gene fragment profiles, which were then displayed on a 6% sequencing gel. Differentially displayed bands were cut from the sequencing gel and extracted with 50 µl water for 15 min in a boiling water bath. The fragments were reamplified with the same set of primers and then electrophoresed on 4% agarose gels. The amplified fragments were recovered from the gels and cloned into a pCRII vector by the

Topo cloning method (Invitrogen, Carlsbad, CA). The vectors were then sequenced and BLAST searches performed with NCBI database to identify genes.

Histological and immunohistochemical analysis

Immunohistochemical staining was performed on both formalin fixed and unfixed frozen 5 μm thick sections on poly-L-lysine slides. For endothelial identification, biotinylated rat anti-mouse CD31 monoclonal antibody (MEC 13.3) was used with fluorescein conjugated streptavidin as the secondary reporting reagent. Rabbit anti-legumain antisera was prepared by immunization with purified human legumain produced in *E. coli* (Choi et al., 1999). This antisera recognized both mouse and rat legumain in frozen sections, as well as human legumain in formalin fixed sections. For staining of legumain in both frozen and formalin fixed sections, rabbit polyclonal anti-legumain antisera was used at 1:500 dilution followed by biotinylated anti-rabbit IgG as the second antibody. The reaction was visualized with Texas-red conjugated streptavidin and the slides were analyzed by laser scanning confocal microscope (Bio-Rad, Hercules, CA). For chromogenic staining, the rabbit polyclonal anti-legumain antibody was followed by a biotinylated goat anti rabbit antisera (Vector, Burlingame, CA). Streptavidin conjugated peroxidase was used and developed with the substrate BAD (Vector, Burlingame, CA).

Western Blot Analysis

Proteins were dissolved in 2× SDS sample buffer for SDS PAGE analysis using gradient (8-16%) Tris-glycine gels. Following electrophoresis, the proteins were transferred to nitrocellulose membranes, and blocked with non-fat milk. The anti-legumain antisera was used as the first antibody and was incubated with membrane for one hour (1:1,000 dilution). The blot was washed three times with PBS, incubated with streptavidin-peroxidase for 15 min and developed by the ECL method (Sigma, St. Louis, MO).

Statistical analysis

Statistical significance of data in this and other Examples was determined by the two-tailed Student's t test.

Results

Over-expression of legumain in solid tumors

Use of restriction fragment differential display (Wrang et al., 2001; Gravesen et al., 2000; Zhang et al., 1998; Theissen et al., 1997) demonstrated that legumain is highly expressed *in vivo* in the CT26 murine colon carcinoma. Immunohistochemical studies of the CT26 tumor indicated that legumain is expressed by both tumor cells and, frequently, by tumor associated endothelial cells, both intracellularly and on the cell surface (FIG. 1A). Legumain over-expression in mouse tumors was confirmed by Western blot analysis. Legumain was also expressed by some normal mouse tissues (FIG. 1B). Legumain expression was not detected in the CT26 cell line in culture that was used to generate the syngeneic mouse colon carcinoma model, and legumain expression was not detected in other tumor cell lines in culture that were tested.

The surprising and unexpected up-regulation during tumor development *in vivo* suggests an *in vivo* environmental response. Legumain appears to be a stress responsive gene, because, although not detectable in cultured cells under typical tissue culture conditions, its expression was markedly elevated in cells subjected to environmental stress, such as serum starvation or *in vivo* growth.

To characterize legumain expression in normal human tissues and tumors, human tumor tissue arrays were analyzed immunohistochemically with anti-legumain antisera (FIG. 1C-F). While legumain expression was sparse in normal tissues, its expression was highest in the kidney. Legumain was also detected in liver and spleen, agreeing with published results (Chen et al., 1997; Chen et al., 1998).

Notably, legumain was highly expressed in the majority of human tumor tissue panels analyzed, which encompassed a wide variety of solid tumors (Table 3). Expression was highest for prostate carcinomas and positive for most breast and colon carcinoma specimens. All central nervous system malignancies were also positive for legumain expression.

Table 3. Legumain detection in human tumors.

Carcinoma Type	Number analyzed	Number positive	Percentage positive	Degree of positivity
Breast carcinoma	43	43	100%	+++
Colon carcinoma	34	32	95%	+++
Lung carcinoma	24	14	58%	+++
Prostate carcinoma	56	42	75%	++++
Ovarian carcinoma	23	17	73%	++
CNS tumors	8	8	100%	++
Lymphoma	14	8	57%	+
Melanoma	12	5	41%	+

Cellular distribution of legumain

Legumain was most abundantly visualized associated within intracellular membranous vesicles (FIG. 2A), consistent with a proposed function as a lysosomal protease. The delivery of membranous vesicles containing proteases, adhesion molecules, and actin binding proteins toward the leading edge of migratory cells has been implicated in cell locomotion (Nabi et al., 1999; Bretscher et al., 1998). The legumain positive membranous vesicles were often concentrated at the invadopodia of tumor cells (FIG. 2B). Unexpectedly, legumain was also observed in apparent association with cell surfaces (FIG. 2C), as well as with the actin cortex (FIG. 2D).

Double staining of legumain+ 293 cells with antibody against integrin β1 and anti-legumain antibody revealed the presence of legumain inside cells in a granular organelle resembling aggregated lysosomes, and also on the cell surface colocalized with β1 integrins (FIG. 2E). The potential binding of legumain to β1 integrins is provocative considering the RGD sequence motif present in legumain, a motif that might facilitate legumain association with cell surface integrins.

Therefore, legumain is highly expressed by most human tumors. A high percentage of breast carcinomas, colon carcinomas, and central nerve system neoplasms strongly expressed legumain, with the highest expression found in prostate tumors. In contrast, legumain was weakly expressed or not observed in the normal tissues of tumor derivation. Furthermore, legumain expression was negative for the cell lines in culture that were used to generate the *in vivo* tumors, even though legumain was readily detected after those cell lines had been placed *in vivo*. These results are indicative of induction of gene expression by the *in vivo* tumor environment.

Example 2: Legumain and Cell Migration, Tumor Invasion, and Metastasis

Example 2 discloses that legumain promotes cell migration and over-expression and is associated with enhanced tissue invasion and metastasis.

Materials and Methods

Cell invasion and mobility assays

Cell migration and invasion assays were performed as described with modifications (Albini et al., 1987). Stock solutions (15mg/ml) of Matrigel basement membrane matrix (Becton Dickinson, Bedford, MA) were stored at -80°C in 100 µl aliquots. After thawing on ice, the stock was diluted 1:50 with cold serum-free culture media and immediately applied to each membrane insert (8 µm pore) that formed the upper chambers of the multi-well invasion assay plate. The Matrigel was incubated overnight in a sterile laminar tissue culture hood. The membranes were hydrated for 2 hours with 250 µl of serum-free medium and excess medium was removed by aspiration. Medium containing 10% FBS was added to the bottom of each well. A suspension of 10^5 cells in 150 µl of serum-free medium was added to the upper chamber and incubated for 12 hours at 37°C, 5% CO_2. At the indicated times, the membrane inserts were removed from the plate and the non-invading cells were removed from the upper surface. Membrane associated cells were stained with 0.09% crystal violet for 30 minutes and washed twice with PBS. The invading cells were counted microscopically. Cell mobility assays were performed in a similar manner except the membrane inserts were not coated with Matrigel, and duration was

shortened. In some assays, protease inhibitors were added to the invasion chamber at the beginning of the incubation.

Zymogram

Control 293 cells and legumain+ 293 cells were plated into 96 wells plates at a cell density of 4,000 cells/well. The cells were allowed to attach overnight, then were serum starved for four hours. Zymogen forms of metalloproteinase 2 or 9 (Chemicon, Temecula, CA) were added at concentration of 0.1 μg/well with 50 μl reaction buffer (39.5 mM citric acid, 121 mM Na_2HPO_4, pH 5.8, 1 mM EDTA, and 0.8% Na_2Cl) and the reactions were continued for 10 minutes. The reactants were collected and mixed with an equal volume of SDS sample buffer and held at room temperature for 10 minutes then applied to a zymogram gel (10% Tris-Glycine gel with 0.1% gelatin substrate). After electrophoresis, the gel was washed briefly and incubated with 2.5% (v/v) Triton X-100 at room temperature for 30 minutes with gentle agitation. Digestion of the incorporated gelatin by activated collagenase was conducted in buffer (50 nM Tris, pH 7.25, 200 mM NaCl, 10 nM $CaCl_2$, 0.05% Brij-35, 0.02% NaN_3) overnight. The gel was stained with Coomassie Blue R250 (Novex, San Diego, CA) and the presence of a protease was readily observed as a clear band.

Results

Legumain expression promotes cell migration and invasion

The effect of legumain expression on cell migration and invasion was investigated. In an *in vitro* migration assay, legumain+ 293 cells exhibited increased migration in comparison with wild type 293 cells. The enhanced migration was inhibited by cystatin, a known inhibitor of legumain protease function, and weakly by TIMP-2 protein, but not by E64 (FIG. 3A).

Next, control 293 cells and legumain+ 293 cells were evaluated in a modified Boyden chamber invasion assay. Legumain+ cells exhibited increased invasion of extracellular matrix. Such increased invasion was inhibited by cystatin (FIG. 3B), but to only a limited extent by TIMP-2. Again E64 was without effect. These experiments were repeated with a 293 cell line in which

the transcription of legumain was conditionally regulated by tetracycline. Comparable results were obtained (data not shown).

Legumain expression correlates with tumor invasion and metastasis

To explore the effects of legumain expression *in vivo*, legumain+ 293 cells and control 293 cells were injected subcutaneously into the backs of WEHI nude mice. Tumors appeared after 2-3 weeks. The initial rates of primary tumor growth were comparable in mice receiving either legumain+ 293 cells or control 293 cells. However, while there was prominent legumain expression in the legumain+ 293 tumors only weak but positive legumain expression was detectable in control 293 tumors, despite an absence of legumain expression by these cells in culture. These results were similar to the observations for the CT26 colon carcinoma cells. Histological analysis of more advanced tumors suggested a lower rate of apoptosis for legumain+ 293 tumors compared to control 293 tumors.

In contrast to control 293 tumors, legumain+ 293 tumors frequently metastasized *in vivo*. At necropsy, 50% of legumain+ 293 tumor bearing mice had metastatic nodules in distant organs (FIG. 4A), predominantly in the lung and liver. In contrast, no metastatic nodules were observed in distant organs within animals that received control 293 cells. The increased invasion and metastasis associated with legumain over-expression is consistent with legumain-facilitated tumor metastasis and progression. The more invasive legumain+ 293 tumors frequently invaded muscles and frequently lacked the well defined pseudo-encapsulation observed with control 293 tumors (FIG. 4B and 4C). This more invasive tumor behavior was evident in early as well as later stage tumors.

Activation of progelatinase A by legumain

Tumor cell surface-associated proteases may degrade extracellular matrix proteins, for example, gelatinase A and cathepsins. For example, the activation of progelatinase A requires cleavage of an asparaginyl bond (Nagase et al., 1997). Therefore a gelatin zymogram study was performed to evaluate whether cell surface bound legumain can convert the 72 kDa zymogen to the 62 kDa active enzyme was examined. Cells expressing legumain, as well as control cells, were deposited in 96 well plates. After attachment and serum starvation,

the culture media was removed. Zymogen forms of gelatinase A and B were incubated with the cells for 10 minutes, and the reaction products were analyzed by zymography. Generation of the 62 kDa active enzyme was observed for cells expressing legumain, and the activation was inhibited by cystatin (FIG. 2F). Legumain alone did not degrade gelatin(data not shown), indicating that legumain expression in cells is needed for such degradation. Moreover, no effect of legumain on zymogen gelatinase B was detected (FIG. 2G).

Discussion

Proteases have been implicated in many aspects of tumor cell biology (Chang et al., 2001). Thus, a protease that is highly expressed by tumor cells or tumor vascular endothelial cells might contribute to tumor cell progression through processing of signaling molecules and their receptors, thereby influencing cellular responses. Such effects might also result in diminished apoptosis (Hanahan et al., 2000), thereby enhancing tumor growth.

Evidence presented herein not only shows atypical expression, but also illustrates participation of legumain in effector functions. In particular, legumain is an apparent regulator of cellular behavior in migration and tissue invasion because cells that highly express legumain exhibited enhanced migratory and invasive properties. A correlation between tumor invasion and metastasis with the presence of cysteine endopeptidases (particularly cathepsins B and L) has been observed (Mai et al., 2000).

Hydrolysis of asparaginyl bonds is prominent in the post-translational processing of cathepsin B, D, and H (Chen et al., 1997; Chen et al., 1998; Yonezawa et al., 1988), which are cysteine proteases. Legumain might therefore activate the local cysteine protease zymogens to form the active two chain protease forms. In addition to the established plasminogen/plasmin system and the metalloproteinase system, a cysteine protease cascade may represent an additional tumor invasion/metastasis cascade. As described, a 62 kDa activated gelatinase A enzyme was observed in cells expressing legumain, and such activation was inhibited by cystatin (FIG. 2F), however legumain had no effect on zymogen gelatinase B (FIG. 2G). Hence, legumain activates the gelatinase A zymogen, an important mediator of extracellular matrix degradation. The

activation mechanism of gelatinase A by legumain differs from that involved with the membrane type matrix metalloproteinases (Itoh et al., 1998). This may be important for tumor cell adaptation to a more invasive and metastatic phenotype.

Legumain-promoted cell migration and invasion can be partially inhibited by cystatin and TIMP-2. The inhibition of mammalian legumain by cystatin is due to a novel second reactive site (Alvarez-Fernandez et al., 1999). Another cysteine inhibitor, E64, has no affect on legumain or cell migration. Hence, the increased cell migration in legumain+ cells is not due to a member of the papain family of cysteine proteases that are characteristically susceptible to E64 inhibition.

Analysis by site-directed mutagenesis of the catalytic residues of mammalian legumain suggests a catalytic dyad exists with the motif His-Gly-spacer-Ala-Cys (SEQ ID NO:1). The presence of this motif is also found in the catalytic sites of the caspases, the aspartate-specific endopeptidases central to the process of apoptosis in animal cells, as well as in the families of clostripain and gingipain which are arginyl/lysyl endopeptidases of pathogenic bacteria. However, legumain is notably distinct from other lysosomal cysteine proteases. In particular, its catalytic activity is unique in that it is the only asparaginyl endopeptidase identified to date. Moreover, the sequence for legumain is conserved through evolution. Its conservation and unique enzymatic activity indicate legumain may have a significant biologic function.

Animal tumor models generated with cells over-expressing legumain had more vigorous and invasive growth and metastasis *in vivo* behavior than similar tumor cells that did not overexpress legumain. These results indicate that the proteolytic function of legumain may activate other protease zymogens. The inhibitory effect of cystatins on tumor cells (Sexton et al., 1997; Coulibaly et al., 1999) is consistent with the involvement of legumain and perhaps other cysteine proteases in tumor invasion and metastasis.

Tumor invasion and metastasis are critical determinants of cancer lethality, linked to 90% of human cancer deaths (Sporn et al., 1996). Invasion and metastasis are considered to be associated properties of tumor cells as they utilize similar processes involving physical attachment of cells to their

environment through cell adhesion molecules (CAMs) and activation of extracellular proteases (Hanahan et al., 2000). Increased expression of proteases and down regulation of protease inhibitors is commonly observed in tumors (Yano et al., 2001; Chamber et al., 1997). Notably, cell surface proteases are often associated with invasive and metastatic tumor cells (Chang et al., 2001). Some proteases are linked to other properties of tumors such as angiogenesis (Stetler-Stevenson et al., 1999) and growth signaling (Werb et al., 1997) as perhaps with legumain.

Protease zymogens are dependent on limited proteolytic activation for conversion to the functional state. Protease cascades are characteristic of many biologic pathways, such as the coagulation, apoptosis, and complement cascades. Similar cascades appear to be involved in tumor invasion and metastasis. Characterization of the later is complicated by the diversity of neoplasms. However, comprehensive profiling of protease expression and function may advance understanding of tumor invasion and metastasis.

Some metalloproteinase inhibitors have demonstrated tumor stasis activities in animal models. Similarly, legumain represents a target for inhibition of growth and metastasis based on results showing that up-regulation of legumain expression is associated with tumor growth and the unique restricted specificity of legumain.

Legumain functions both extracellularly and intracellularly. Therefore, a cell-permeable inhibitor might extend the efficacy observed with cystatin, as the latter is cell-impermeable and has shown limited inhibition of *in vitro* cell migration and invasion.

Tumor cells with higher legumain levels appear to be more resistant to apoptosis. Although the precise molecular pathway has yet to be defined for this effect, lysosomal proteases are known to participate as effector enzymes in apoptosis (Foghsgaard et al., 2001; Castino et al., 2002). Thus, the sub-cellular localization of legumain may determine its targets and thereby its effects on the apoptosis cascades.

Example 3: Tumoricidal Effects of a Prodrug

Legumain's unique functional properties and high level of expression in a wide range of human tumors makes it a potential candidate target for enzymatic activation of a prodrug that can help eradicate tumors.

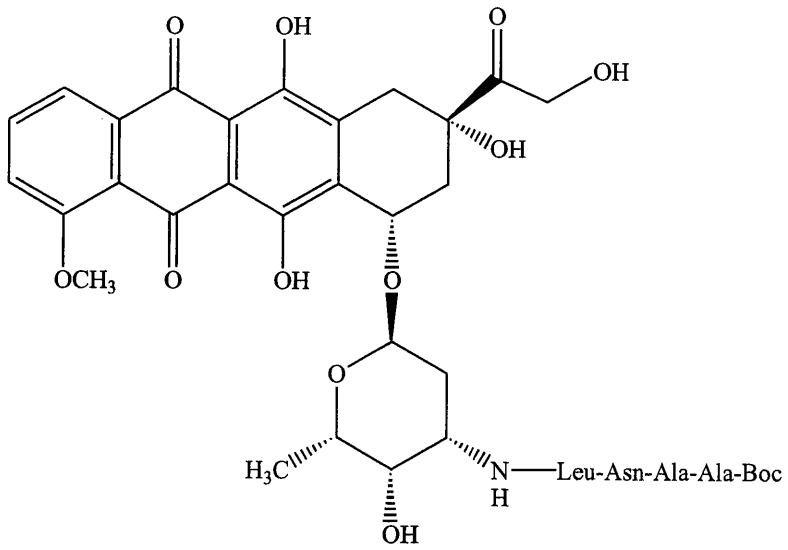
The integrity of the amino group of doxorubicin is essential for function. It has been shown that doxorubicin tolerates the addition of a leucine residue at this site. However incorporation of additional amino acids abolishes cytotoxic activity (de Jong et al., 1992; Denmeade et al., 1998).

In this Example, a prototype prodrug was synthesized by addition of an asparaginyl endopeptidase substrate peptide to doxorubicin. Upon exposure to legumain, the agent was converted to an active cytotoxic leucine-doxorubicin molecule. The prodrug had markedly reduced toxicity compared to doxorubicin, but it was effectively tumoricidal in a murine colon carcinoma model where it was presumably cleaved to form the leucine-doxorubicin cytotoxin. Therefore, according to the invention, legumain is a new target for tumoricidal prodrug development and therapy.

Materials and Methods

Prodrug Synthesis

N–(–*t*-Butoxycarbonyl-L-alanyl-L-alanyl-L-asparaginyl-L-leucyl)doxorubicin (SEQ ID NO:7) has the following structure.



Note that the amino acid sequence shown above is written with the C-terminus to the left and the N-terminus to the right. Thus, the doxorubicin moirty is attached to the C-terminus of the peptide.

N–(–*t*-Butoxycarbonyl-L-alanyl-L-alanyl-L-asparaginyl-L-leucyl)doxorubicin (SEQ ID NO:7) was synthesized as follows. To cold (0°C) solution of *t*-Butoxycarbonyl-L-alanyl-L-alanyl-L-asparaginyl-L-leucine (43 mg, 95 μmol) and 4-Methylmorpholine (20 μL, 200 μmol) in 5 mL DMF was added O-Benzotriazol-1-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU) (54 mg, 142.5 μmol). After 10 min, doxorubicin hydrochloride (50 mg, 86 μmol) was added and the mixture was stirred for 2 h at room temperature in the dark. The solution was diluted with 30 mL of EtOAc and washed with water. The solvent was evaporated, and solids were dried over $MgSO_4$ and purified over silica gel using $CHCl_3$/MeOH (90/10) while protected from light to yield 65 mg of compound **1** (75% yield). ^{1}H NMR (600 MHz, CD_3OD, δ): 0.82 (d, 3H, J = 6.1), 0.88 (d, 3H, J = 6.6), 1.28-1.35 (m, 9H), 1.43 (s, 9H), 1.59-1.74 (m, 4H), 2.05 (m, 1H), 2.17 (m, 1H), 2.36 (d, 1H, J = 14.5), 2.67 (m, 1H), 2.79 (m, 1H), 2.91 (d, 1H, J = 18.0), 3.04 (d, 1H, J = 18.0), 3.62 (m, 1H), 4.01-4.04 (m, 4H), 4.11 (m, 1H), 4.22-4.32 (m, 3H), 4.59 (dd, 1H, J = 5.9, 7.2), 4.74 (d, 2H, J = 4.4), 5.08 (s, 1H), 5.39 (d, 1H, J = 3.1), 7.51 (d, 1H, J = 8.8), 7.78 (dd, 1H, J = 7.9, 7.9), 7.86 (d, 1H, J = 7.5). Preparation HRMS (MALDI) calculated for $C_{48}H_{64}N_6O_{18}$ $[M+Na]^+$ is 1035.4169, and found is 1035.4234. The compounds were purified by semi-preparative HPLC.

Cytotoxic assays

The WST-1 cell proliferation reagent (Roche Molecular Chemicals, Germany) was used to determine cell proliferation by quantification of cellular metabolic activity. Control 293 cells and legumain+ 293 cells were cultivated in microtiter plates (5 x 10^3 cells per well in 100 μl) and were incubated with serial concentrations of legubicin or doxorubicin for 48 h. Subsequently, 10 μl of WST-1 solution (1 mg/ml WST-1, 25 μM –methyldibenzopyrazine methyl sulfate) was added per well, and mixtures were incubated for an additional 4 h. The tetrazolium salt WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disfonate) was cleaved by the mitochondrial succinate-tetrazolium-reductase system to formazan in cells which directly correlates to the

number of metabolically viable cells in the culture. The amount of formazan salt was quantified in three replicates by absorbance at 450 nm using a micro-plate reader (Molecular Devices, Palo Alto, CA). All results were derived from replicate experiments with similar results.

Animal models

The CT26 syngeneic murine colon carcinoma model was generated and maintained in The Scripps Research Institute animal facility. Balb/C mice aged 4 to 6 weeks from the breeding colony were inoculated with 500,000 syngeneic CT26 tumor cells per site subcutaneously in the back. Treatment was initiated when the tumors reached 4 mm in diameter through bolus intraperitoneal injection of the indicated reagents. Treatment was repeated at 2 day intervals. The human 293 tumor models were generated in WEHI nude mice (The Scripps Research Institute breeding colony). Either legumain+ 293 cells or control 293 cells (10^6 cells/site) were inoculated subcutaneously into the backs of mice. Tumor growth and physical signs were monitored daily including any gross evidence of tumor necrosis, local tumor ulceration as well as evidence of toxicity including mobility, response to stimulus, eating, and weight of each animal. These procedures have been reviewed and approved by the Institutional Animal Care and Use Committee at The Scripps Research Institute. The work was conducted in The Scripps Research Institute facilities which are accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. The Scripps Research Institute maintains an assurance with the Public Health Service, is registered with the United States Department of Agriculture and is in compliance with all regulations relating to animal care and welfare.

Results

Model prodrug activation by legumain

The functional capacity of tumor cell-associated legumain was explored based on the novel asparaginyl specific endopeptidase activity of legumain. The amino group of doxorubicin is critical for function. However a leucine residue can be added with retention of cytotoxicity. The prodrug, N–(–*t*-Butoxycarbonyl-L-alanyl-L-alanyl-L-asparaginyl-L-leucyl) doxorubicin (SEQ ID NO:7), was synthesized by addition of an asparaginyl endopeptidase substrate

peptide Boc-Ala-Ala-Asn-Leu to the amino group of doxorubicin through a peptide bond at carboxy terminus of leucine. Upon cleavage by legumain, the prodrug is converted to a leucine-doxorubicin molecule, thereby regaining cytotoxic function. In addition, the presence of the Boc at the amino terminus prevents aminopeptidase hydrolysis of the peptidyl component. This prodrug is designated Legubicin (FIG. 5A).

First, the cytotoxic activity of doxorubicin and legubicin upon activation by legumain was analyzed *in vitro* using legumain+ 293 cells and control 293 cells (FIG. 5B). The effect of doxorubicin on both 293 cell types was similar, with legumain+ cells only slightly more resistant to doxorubicin. In contrast, the cytotoxic effect of legubicin on control 293 cells was less than 1% of that of doxorubicin, indicating peptide conjugation to form the prodrug had abolished the cytotoxic effect of the doxorubicin. The dose responsive curve of legubicin on control 293 cells was parallel to that of doxorubicin on both 293 cells, suggesting that the residual cytotoxicity of legubicin may result from slight (~1%) doxorubicin contamination. In contrast, a profound cytotoxic effect of legubicin was observed for legumain+ 293 cells. The dose response curve of legumain+ cells challenged with legubicin also differed from that for these cells exposed to doxorubicin.

Tumoricidal effect of legubicin *in vivo*

The *in vivo* effects of legubicin on normal and tumor bearing hosts and efficacy in tumor eradication was investigated using the CT26 murine syngeneic colon carcinoma model. Legubicin was very well tolerated in mice with much reduced toxicity compared to doxorubicin. Intraperitoneal injection of legubicin at a dosage of 5 mg/kg three times at 2 day intervals induced complete growth arrest of the tumors with little evidence of toxicity (FIG. 6A1-6A3), as most readily evidenced by the absence of weight loss (FIG. 6A3). In contrast, doxorubicin failed to produce similar anti-tumor efficacy at doses approaching its maximum tolerable dose (MTD). When doxorubicin was administered by the same protocol and dosage as for legubicin, toxicity was fatal.

A single injection of 5 mg/kg legubicin induced more profound tumoricidal effects than animals given a comparable dose of doxorubicin (FIG. 6B and 6C), as observed by histology. TUNEL assay analysis of tumor tissues

revealed a higher apoptotic index for legubicin than for doxorubicin treatment (FIG. 6D and 6E). Surprisingly, in organs that do express legumain, such as kidney and liver, no injury was evident (not shown). These observations indicate that legubicin has significantly improved safety and therapeutic indices compared to doxorubicin.

Discussion

The high level of legumain expression by tumor cells, coupled with its unusual and highly specific substrate requirement for catalytic function, makes it an attractive candidate for prodrug conversion in a therapeutic mode. Current cancer chemotherapeutic agents have significant undesirable cytotoxicity. A promising approach to increase therapeutic delivery to tumor cells is to exploit enzymes that are more highly expressed by tumor cells and thereby achieve local prodrug activation to the active compound. Peptide conjugates of doxorubicin designed for activation by plasmin (de Groot et al., 1999; Chakravarty et al., 1983) and cathepsins (Satchi et al., 2001; Dubowchik et al., 1998a and 1998b) have been suggested. However, those conjugates are relatively deficient in target selectivity since plasmin generation is not tumor selective.

The doxorubicin prodrug exemplified herein was synthesized by incorporating a peptide extension to the amino group of doxorubicin. This agent, designated legubicin, was analyzed for cytotoxicity on cells not expressing legumain where it was less than 1% as toxic as doxorubicin. However, on cells expressing legumain, legubicin was profoundly cytotoxic, consistent with its conversion to leucine-doxorubicin.

Intraperitoneal administration of legubicin at 5mg/kg resulted in complete arrest of tumor growth without identifiable toxicity, such as weight loss, in contrast to doxorubicin treated mice. Legubicin administration produced profound tumor cell apoptosis as indicated by TUNEL assay. Unexpectedly, in organs containing cells that normally express legumain, such as kidney and liver, no injury was evident. Thus, legumain activation of this prodrug may require conditions not present in normal tissue. Prodrug activation may be carried out by secreted or cell surface associated legumain; whereas legumain appear to be localized in lysosomal vesicles in normal tissues. Legumain requires an acidic

environment for optimal catalytic activity, which may not be present in normal tissues. Legubicin also appears to have an improved therapeutic index compare to its parent doxorubicin. Whereas clinical use of doxorubicin is limited by its toxicity, a prodrug that preserves activity, but that has reduced toxicity is an attractive alternative.

Example 4: Analysis of substrate specificity of legumain using phage displayed substrate libraries.

Legumain demonstrated an uniquely restricted specificity requiring an asparagine residue at the P1 position. However it does not hydrolyze all asparaginyl carboxyl peptide bonds of all protein substrates. Chen, J.M., et al., *Cloning, isolation, and characterization of mammalian legumain, an asparaginyl endopeptidase.* J Biol Chem, 1997. **272**(12): p. 8090-8; 47; Mathieu, M.A., et al., *Substrate specificity of schistosome versus human legumain determined by P1-P3 peptide libraries.* Mol Biochem Parasitol, 2002. **121**(1): p. 99-105. Many asparagine residues may not be accessible and able to form transition state analogues in most proteins. Amino acid preferences for parasite legumain indicate legumain has some degree of preference for the P2 and P3 positions, however it can accept all residues except proline at the P1' position. Mathieu, et al. (2002); Schwarz, G., et al., *Characterization of legumain.* Biol Chem, 2002. **383**(11): p. 1813-6.

The substrate specificity of mammalian legumain will be explored using combinatorial phage display peptide libraries that will be constructed by inserting a legumain recognition site between a Tissue Factor extracellular domain (TF_{1-218}) and fusion with a second gene III in M13 phage. TF_{1-218} is particularly resistant to proteolysis. The legumain substrates library will contain two random amino acids flanking each side of an asparagine residue (XXNXX). Such a library has a modest library size of 1.6×10^5 different sequence combinations. DNA encoding these peptide sequences will be synthesized and cloned into the phage vector display TF_{1-218} as a fusion protein with the second phage gene III coat protein.

The phage displayed substrate library will be immobilized in 96 well plates through anti-TF antibody, and recombinant legumain expressed by Pichia

yeast will be used for proteolysis to release the susceptible phage from the plate. These phage will be plated and amplified. Individual phage will be selected and sequenced to detect the susceptible residues surrounding the asparagine residue. The motifs of highest frequency represent the more favorable sequences for legumain recognition. The identified peptide sequences will be confirmed with synthetic peptides and its binding kinetics will be characterized.

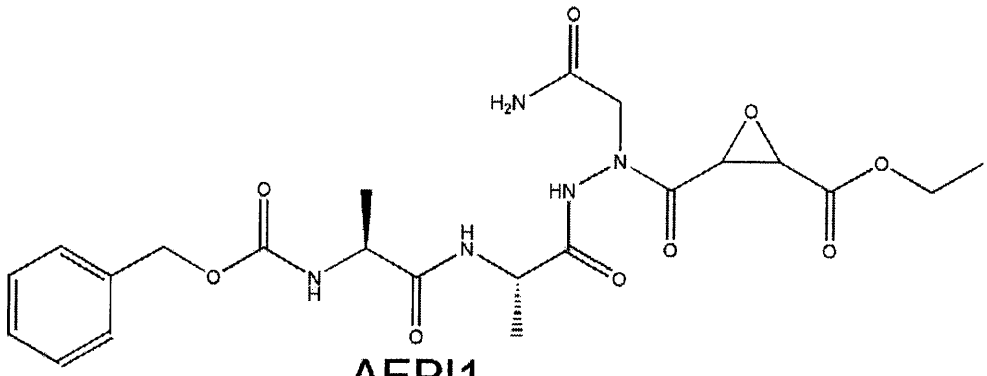
Example 5: Hypoxia Induces Legumain Expression and Localization of Legumain on the Cell Surface Is Needed for Tumor Invasion and Angiogenesis.

This Example illustrates that inhibition of legumain not only inhibits tumor cell invasion but also inhibits angiogenesis by tumor cells.

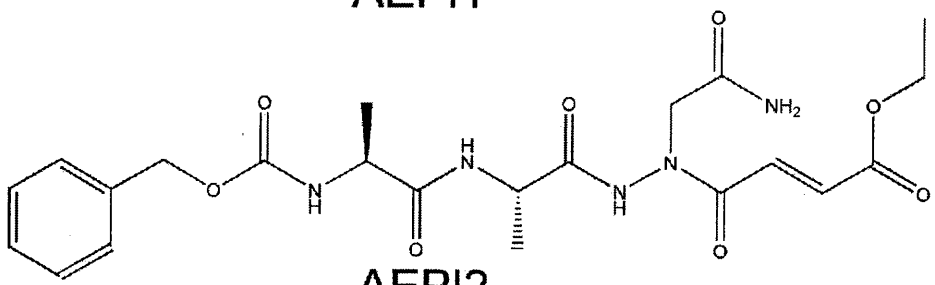
Materials and Methods:

The materials and methods employed are generally described in the other Examples provided in this application.

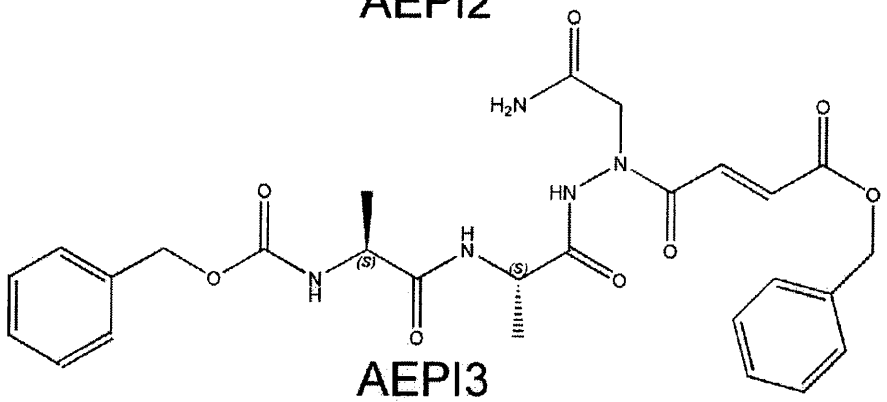
The four AEPI's employed in some of the experiments described herein have the following structures: AEPI-1 is Cbz-Ala-Ala-AzaAsn-(S,S)-EPCOOEt; AEPI-2 is Cbz-Ala-Ala-AzaAsn-CH=CH-COOEt; AEPI-3 is Cbz-Ala-Ala-AzaAsn-CH=CH-COOBzl; and AEPI-4 is Cbz-Ala-Ala-AzaAsn-CH=CH-CON(CH3)Bzl. The structures of these inhibitors are shown below:



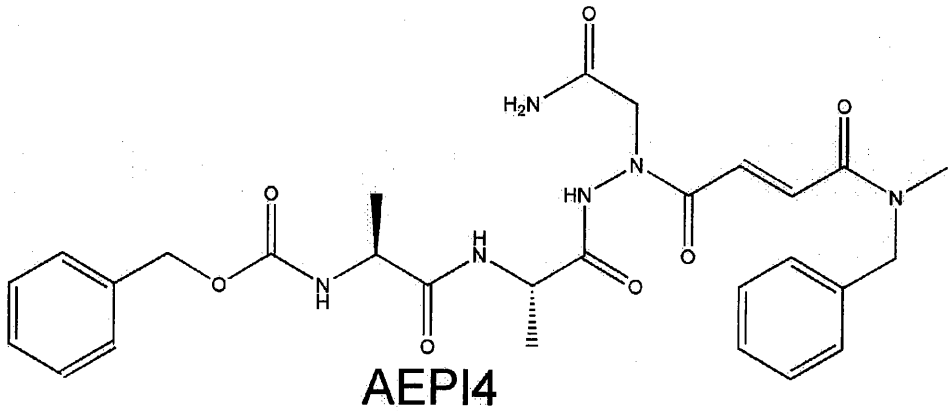
AEPI1



AEPI2



AEPI3



AEPI4

5

Results

Legumain expression is induced by hypoxia. Legumain expression in human tumor cells and endothelial cells was examined by western blot analysis under both normoxic and hypoxic culture conditions, because hypoxia occurs early in the development of primary and metastatic tumors. In both AMD-MB231 human breast cancer cells and MB21 human melanoma cells, legumain expression was induced in cells cultured under hypoxic conditions (1% O_2), and its level continued to rise as the duration hypoxic exposure increased (FIG. 7A). HUVECs expressed very little legumain under normal culture conditions, but legumain expression was significantly enhanced when these cells were cultured under hypoxic conditions for 72 hours (FIG. 7A).

The 4T1 metastatic mouse mammary carcinoma metastasizes spontaneously to lung (FIG. 7B). When legumain expression was examined in lung metastatic sites, such expression was observed very early in the development of metastatic tumors. Legumain expression was observed when even only a small number of tumor cells were present in metastatic sites (FIG. 7C). These data indicate that up-regulation of legumain expression occurs early during tumor development.

$\alpha_v\beta_3$ integrins are cell surface receptors for legumain. Legumain is distributed intracellularly and is presented on cell surfaces in the tumor microenvironment (see previous Examples). However, legumain contains a RGD domain that is usually present in proteins that bind to integrins. To determine whether legumain binds to integrins, immunoprecipitation experiments were performed using a panel of anti-integrin antibodies on MDA-MB231 cell lysates, because these cells express low level of legumain in culture. Legumain was co-precipitated with anti-αv, αvβ3, β3, and β1 antibodies (FIG. 8A). These data suggest that αvβ3 integrin binds to legumain.

To further characterize the interaction between legumain and integrin, co-immunoprecipitation was performed with anti-legumain antibodies and detection was performed with anti-αv integrin antibodies (FIG. 8B). Again, legumain was detected when anti-αvβ3 antibodies were used as the immunoprecipitating antibody (FIG. 8B).

Next, immunohistochemical staining of MDA-MB-231 human mammary carcinoma cells was performed with both anti-αvβ3 and anti-legumain antibodies (FIG. 8C). Very little legumain and αvβ3 integrin was expressed in cells cultured under normal conditions. However, the levels of legumain and αvβ3 integrins were both dramatically elevated under hypoxic conditions. These experiments also demonstrated that legumain and integrin were transported to cell surfaces where these proteins co-localized extensively (FIG. 8D). In migrating cells, the legumain:αvβ3 complex are predominantly present at the leading edge of the cells (FIG. 8C).

$\alpha_v\beta_3$ integrin is a co-factor promoting legumain proteolytic activity. The effects of integrin binding to legumain enzymatic activity was determined by observing hydrolysis of a substrate of legumain to generate a fluorescent product. FIG. 9A shows that as the concentration of αvβ3 integrin increases legumain amidolytic activity dramatically increases. These data suggest that αvβ3 integrin is not only a cell surface receptor of legumain, it is also a co-factor that increases legumain activity. In particular, binding of legumain to αvβ3 integrin can increase legumain activity nearly 100 fold (FIG. 9B).

In addition, binding to αvβ3 also affects the pH dependency of legumain (FIG. 9C). The activity of legumain: αvβ3 complexes were measured under different pH conditions and compared to that of uncomplexed legumain. As shown in FIG. 9C, binding of legumain to αvβ3 integrin shifts its peak activity from pH 5.2 to pH 6.0. Typically free legumain is inactive at pH 7.0 (FIG. 9C). However, unlike free legumain, the legumain: αvβ3 integrin complex was still active at pH values that were almost as high as pH 7.0 (FIG. 9C). There, integrin binding significantly increases legumain activity at the mildly acidic conditions that exist in the extracellular space of the tumor microenvironment. Moreover, the cell-surface location, the enhanced amidolytic activity of integrin-bound legumain and the shift in pH dependency indicates that the complex between legumain and αvβ3 integrin is likely the primary site in the tumor microenvironment where the prodrugs of the invention are activated.

Asparaginyl endopeptidase activity of legumain is critical for angiogenesis. The activity of legumain:αvβ3 against the physiological substrate of legumain (metalloproteinase-2, MMP2) was then evaluated. As shown in FIG.

9D, legumain not only removes the pro-MMP2 propeptide but also cleaves MMP2 between its proteolytic domain and its integrin binding hemopexin domain (FIG. 9E). Therefore, the legumain: αvβ3 complex not only activates MMP-2 but it also generates a hemopexin fragment, which is a known inhibitory molecule for angiogenesis (FIG. 9D-E). These results indicate that the legumain:αvβ3 complex is an important modulator of pericellular proteolysis during tumor invasive growth and angiogenesis.

To further evaluate the role of asparaginyl endopeptidases in angiogenesis, four asparaginyl endopeptidases inhibitors (AEPI) were tested using recombinant legumain in an amidolytic activity assay with fluorescent substrate. FIG. 10A graphically illustrates the effects that AEPI-1 to AEPI-4 (also referred to as legumain inhibitors LI-1 to LI-4) have on asparaginyl endopeptidase activity. Both AEPI-1 and AEPI-2 had IC_{50} values of about 20 nM and 21 nM, respectively. The IC_{50} value for AEPI-3 was 34 nM, while the IC_{50} value of AEPI-4 was 158 nM. The cytotoxicity of AEPI-1 was also assessed in wild type 293 cells (CC_{50}, 320 μM) and in 293 cells that recombinantly expressed legumain (CC_{50}, 430 μM). No apparent cytotoxicity was observed until almost 100 μM AEPI-1 was used in these assays (FIG. 10B).

The effect of AEPI-1 on endothelial cell function was assessed *in vitro* in a matrigel endothelial cell tube formation assay. AEPI-1 suppressed HUVEC tube formation in a dose dependent manner (FIG. 10C). Inhibition of tube formation was apparent at AEPI-1 concentrations of 100 nM and was completely inhibited when 600 nM AEPI-1 was used. In contrast, addition of recombinant legumain dramatically promoted and accelerated tube formation (FIG. 10D). HUVEC tube formation was observed as early as 5 hours after assay initiation when legumain was added. Under normal conditions 24 hours were required for the vascular tubes to form. AEPI-1 also inhibited hypoxia induced tube formation and endothelial cell invasion in an invasion assay (FIG. 10E and 10F).

The effects of AEPI-1 on angiogenesis were further evaluated in mouse aortic sprouting assays. First, AEPI-1 was added to the beginning of the experiment to determine its effect on initiation of vessel sprouts (FIG. 11A). AEPI-1 inhibited FGF-2 induced vessel sprouts in a dose dependent manner (FIG. 11A). In the second assay, AEPI-1 was added at the third day after aortic

sprouts were already formed (FIG. 11B), to ascertain whether asparaginyl endopeptidase activity was required for continued extension of vessel growth. Addition of AEPI-1 after sprouts were already formed significantly reduced extension of established vascular outgrowth (FIG. 11B). These data suggest that legumain has a continuing role in vascular formation throughout vessel development. These data also suggest that legumain's activation of proteases and its association with integrins may play an important role in angiogenesis.

The effect of AEPI-1 on angiogenesis was further investigated in vivo using a Matrigel plug assay. Matrigel containing FGF-2 was injected subcutaneously into Balb C nu/nu mice. When AEPI-1 was present, FGF-2 induced vessel formation was significantly inhibited in the plug (FIG. 11C) as indicated by the gross appearance, hemoglobin content, and histology (FIG. 11D) of the plug.

The effect of AEPI-1 on angiogenesis induced by cancer cells was then investigated by including human breast cancer cells (MDA-MB-231 cells) in the Matrigel plugs. The cancer cells produced a spectrum of angiogenic factors and induced robust angiogenic vessel growth (FIG. 11C and 11D). However, this cancer cell-induced angiogenesis was essentially completely inhibited by AEPI-1 (FIG. 11C and 11D). These data indicate that inhibition of asparaginyl endopeptidase activity affects angiogenesis even when such angiogenesis is induced by a wide range of angiogenic factors.

AEPI-1 inhibits tumor invasive growth. The function of legumain in tumor invasion was then examined using an invasion assay that included recombinant legumain protein, which promoted cancer cell invasion. The legumain-promoted cell invasion was partially inhibited by cystatin (FIG. 12A). These data are consistent with a role for legumain in activating gelatinase A and cathepsins that are implicated in extracellular matrix degradation.

The effect of AEPI-1 on tumor growth and invasion was then evaluated in a human mammary carcinoma model established in Balb C nu/nu mice. The MDA-MB-231 cells grew aggressively in vivo and formed a tumor that was similar to a human infiltrating ductal carcinoma with apparent glandular

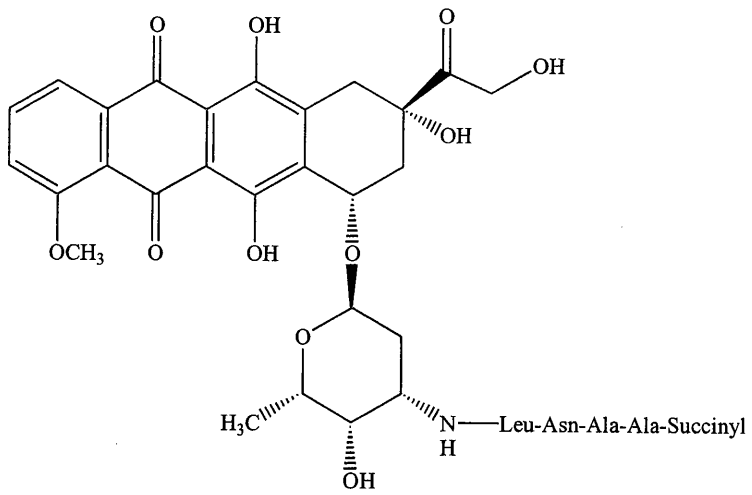
differentiation. The tumor locally invaded surrounding tissues and skeletal muscles.

As shown in FIG. 12C, AEPI-1 not only completely suppressed tumor growth, it also blocked the tumor differentiation into a more invasive form. Two weeks after tumor cell implantation, the treated tumors failed to grow, while the untreated control tumor developed into sizable invasive tumors.

Collectively, these data indicate that the legumain:αvβ3 complex has a significant role in tumor development and further show that asparaginyl endopeptidase inhibitors are effective in suppressing angiogenesis and tumor invasive growth. Therefore, asparaginyl endopeptidase inhibitors are promising new candidates for cancer therapy.

Example 6: Cell-impermeable Prodrug Eradicates Multiple Drug Resistant Neoplasms

This Example shows that LEG-3 is a cell-impermeable pro-drug with reduced toxicity and markedly enhanced efficacy relative to the cell impermeable doxorubicin (Dox). The structure of LEG-3 is shown below as formula IB.



Material and Methods

Reagents and cell lines. Rabbit polyclonal antisera against human legumain as well as 293 cells stably expressing human legumain were kindly

provided by Dr. D. Roodman (Department of Medicine and Hematology, University of Texas Health Science Center, San Antonio, TX). A legumain substrate peptide was purchased from Bachem, Inc (King of Prussia, PA). Doxorubicin was purchased from Sigma (St. Luis, MO) and DMEM media was from Invitrogen (Carlsbad, CA). The CT26 murine colon carcinoma, C1300 mouse neuroblastoma cell lines, and the human HT1080 fibrosarcoma cells were purchased from ATCC.

Antibody preparation. Anti-legumain antibodies were prepared by immunizing rabbits with KLH conjugated peptide CGMKRASSPVPLPP (SEQ ID NO:16). A cysteine is added to the legumain sequence. The anti-legumain antibodies were affinity purified from resultant antisera using peptide antigen coupled to Ultralink Iodoacetyl Gel from Pierce (Rockford, IL). The bound antibodies were eluted by glycine buffer (100 nM, pH 2.7) and neutralized immediately by adding one-tenth volume of 1 M Tris pH 7.5.

Western blot. Proteins were dissolved in 2xSDS sample buffer for SDS-PAGE analysis using gradient Tris-glycine gels (8–16%). After electrophoresis, the proteins were transferred to nitrocellulose membranes and blocked with nonfat milk. The anti-legumain antiserum was used as the first antibody and was incubated with the membranes for 1 hr (1:1,000 dilution). The blot was washed three times with PBS, incubated with strepavidin-peroxidase for 15 min, and developed by the enhanced chemiluminescence method (Sigma, St. Louis, MO).

Flow cytometry analysis. Single cell suspensions were prepared from organs and tumor tissues as reported by Liu et al. Cancer Res. 63: 2957-64 (2003). Rabbit anti-Legumain antisera diluted 1:5,000 or antigen purified anti-legumain antibody at 0.5 μg/ml in PBS are used to detect legumain. This is followed by FITC-conjugated goat anti-rabbit IgG diluted 1:5000 in PBS (BD Pharmingen, La Jolla, California). For CD14 staining, the PE conjugated anti-mouse CD14 antibody diluted 1:3000 in PBS was used (BD Pharmingen, La Jolla, California).

Immunohistochemical analysis. Immunohistochemical staining was performed on 5-μm thick frozen sections on poly-L-lysine slides. For endothelial identification, biotinylated rat anti-mouse CD31 monoclonal antibody (MEC 13.3) was used with Texas red conjugated strepavidin as the

secondary reporting reagent. For staining of legumain, rabbit polyclonal anti-legumain antisera was used at 1:500 dilution or antigen purified anti-legumain polyclonal antibody at 0.5 μg/ml, and visualized with FITC conjugated goat anti-rabbit antibody. For the identification of tumor associated microphage, rat anti-mouse CD68 antibody was used and followed by an anti-rat antibody conjugated with Texas red. For identification of collagen I, a biotinylated rabbit anti-mouse collagen I antibody was used at 1:250 dilution and visualized Texas red conjugated strepavidin. The slides were analyzed by laser scanning confocal microscope (Bio-Rad, Hercules, CA).

Prodrug synthesis. The synthesis of the succinyl version of the prodrug utilized the azide method to protect the peptide from racemization. In principle, the N-protected amino acids or peptide-esters are converted by hydrazine derivatization to an acid hydrazide. Subsequent reaction with HNO_2 or derivatives leads to anacylazide. Thus, the Succinyl-Ala-Ala-Asn-Leu-N_2H_3 (SEQ ID NO:) peptide was prepared by using liquid phase synthesis. It was directly used to synthesize the target compound. An example of the synthesis is as follows: Solution A: 1040 mg Succinyl-Ala-Ala-Asn-Leu-$N_2H_4^+F^-$ (SEQ ID NO:) was dissolved in a small amount of DMF cooled to -10°C, and 1.5 ml of 4 N HCl, dioxane was added followed by 2.1 mM isoamylnitrite. The mixture was stirred for 30-40 min at -10°C and then the pH carefully adjusted to 7.5 with diisopropyl-ethylamine. Solution B: 1210 mg doxorubicin acetate was dissolved in a small amount of DMF at room temperature, the pH adjusted to 7.5 with DIPEA and then chilled to -10°C. Solution A and solution B were combined, the pH readjusted to 7.5 and monitored throughout the reaction. The reaction mixture was allowed to warm to 4°C, and allowed to stand overnight. HPLC analyses indicate approximately 80% completion of the reaction within 24-48 hrs. The reaction mixture was then diluted 10-fold with 0.1% TFA (in H_2O) and applied directly onto preparative HPLC. A linear acetonitrile gradient was used to elute the target compound. Fractions were analyzed for purity, combined, and lyophilized. HPLC, AAA, MS analyses were performed on the lyophilized powder.

Cytotoxicity assays. The WST-1 cell proliferation reagent (Roche Molecular Chemicals, Germany) was used to determine cell proliferation by

quantization of cellular metabolic activity. Control 293 cells and legumain$^+$ 293 cells were cultivated in microtiter plates (5 x 10^3 cells per well in 100 µl) and were incubated with serial concentrations of LEG prodrug or Dox for 48 h. Subsequently, 10 µl of WST-1 solution (1 mg/ml WST-1, 25 µM – methyldibenzopyrazine methyl sulfate) was added per well, and mixtures were incubated for an additional 4 h. The tetrazolium salt WST-1 (4-[3-(4-lodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disfonate) was cleaved by the mitochondrial succinate-tetrazolium-reductase system to formazan in cells in direct correlation with the number of metabolically viable cells in the culture. The amount of formazan salt was quantified in three replicates by absorbance at 450 nm using a micro-plate reader (Molecular Devices, Palo Alto, CA). All results were derived from replicate experiments with similar results.

Dox and LEG-3 uptake assay. Legumain$^+$ 293 cells or control 293 cells (2.5 x 10^5 cells/well) were seeded in six-well plates. The culture plates were then incubated for 24 h at 37°C and 5% CO2, and the medium in each well was replaced with 2 ml of serum-free, antibiotic-free medium containing various concentrations of Dox or LEG-type prodrug compounds. The cells were incubated 1.5 h then washed three times with 2 ml of cold PBS. At this point, cell nuclear positivity for Dox could be analyzed by fluorescent microscopy. For quantitative assays the cells were then lysed by adding 0.5 ml of water and gently rotated on an orbital shaker for 5 min at room temperature. The lysed cells were added to 1.5 ml acidified ethanol, and incubated at 4°C in the dark for 3 h. Total Dox and LEG content was measured fluorometrically using a Perkin-Elmer LS-50-B spectrofluorometer (excitation: 470 nm; emission: 590 nm). Fluorescence intensity was translated to drug concentration by use of a standard curve prepared from Dox and LEG solutions in cell lysates that were not previously exposed to the drug. Results are expressed as the mean $\pm$ SD of at least three replicates for each experiment.

Determination of marrow toxicity. Groups of healthy Balb C mice (n=4) were injected *i.p.* with a single dose of LEG-3 (49.4 µmol/kg or 4.94 µmol/kg) or Dox (3.4 µmol/kg). On day 7, retro-orbital sinus blood samples

were collected into 10 mM EDTA, and counted by hemocytometer after lysis of red blood cells with an acidified methyl violet solution.

Determination of Maximum Tolerated Dosage (MTD). Four six-week-old Balb/c mice were used for each experimental group. The mice were weighed individually and the average weight of the group is used to determine the exact doses. Mice were given i.p. injection once a day for five days. The MTD is defined as the maximum drug dose administered to non-tumor-bearing mice once daily for five consecutive days without mortality.

Tissue distribution. LEG compounds or Dox was injected i.p. into mice, 12 h later the animals were perfused and the Dox autofluorescence was measured following homogenization in 50% ethanol and then diluted with an equal volume of 50% ethanol containing 0.6 M HCl. Fluorescence measurements were obtained with excitation at 470 nm and emission at 590 nm, concentrations were derived by conversion from a Dox standard curve. Tissues from saline injected mice provided controls. Blood samples were to 0.75 ml with PBS, centrifuged, the pellets washed with PBS and Dox extracted with ethanol, 0.3 M HCl.

Animal models. The CT26 syngeneic murine colon carcinoma model was generated and maintained in The Scripps Research Institute animal facility. This model was produced in Balb/c mice aged 4 to 6 weeks. Mice were injected with $5x10^5$ CT26 tumor cells per subcutaneous site on the back. The C1300 mouse neuroblastoma model was generated in A/J mice by subcutaneous injection of $5x10^5$ C1300 cells per site on the back. Treatment was initiated when the tumors reached 4 mm in diameter through bolus intraperitoneal (syngeneic tumors) or intravenous (human tumors) injections of the indicated reagents. Treatment was three times per week for two weeks. The human HT1080 fibrosarcoma was xenografted in Balb/c nu/nu mice obtained from The Scripps Research Institute breeding colony; and HT1080 cells ($1x10^6$ cells/site) were inoculated subcutaneously into the backs of mice. The MDA-PCa-2b human prostate carcinoma model was generated in WEHI nude mice and these cells (10^6 cells) were also injected subcutaneously. Tumor growth and signs of physical discomfort were monitored daily. Such signs included any gross evidence of tumor necrosis, local tumor ulceration as well as evidence of toxicity

including changes in the animal's mobility, response to stimulus, piloerection, eating, and weight. These procedures have been reviewed and approved by the Institutional Animal Care and Use Committee at The Scripps Research Institute. All the experiments were conducted in The Scripps Research Institute facilities which are accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. The Scripps Research Institute maintains an assurance with the Public Health Service, and is registered with the United States Department of Agriculture and is in compliance with all regulations relating to animal care and welfare.

Statistical analysis. Statistical significance of data was determined by the two-tailed Student's *t* test, except for statistical significance of survival curves, which used the Logrank Test using GraphPad Prism version 3.00 (GraphPad Software, San Diego, CA).

Results

Legumain is highly expressed by cells in the tumor microenvironment. As shown above, legumain is expressed *in vivo* by tumor cells and proliferating endothelial cells intracellularly as well as on their cell surfaces. However, legumain is not detectable in or on CT26, C1300, HT1080, and MDA-PCa-2b as well as eight other tumor cell lines in culture, the same cells used to generate a set of legumain expressing tumors *in vivo* (FIG. 13A). Further, the legumain endopeptidase is not detectable in large scale survey panels of tumor cell lines in culture, except for THP-1 cells, based on a search of the NCI tumor cell expression database.

Using immunohistochemical analysis, legumain is usually present in tissue sections of human colon carcinomas, neuroblastomas, fibrosarcomas, and prostate carcinomas, representative of the types of neoplasms investigated in the present study. Legumain expression was not detectable in the normal tissues of origin for these neoplasms. The remarkable local expression of legumain not only by neoplastic cells but also by stromal cells in association with tumor development *in vivo* suggests that such expression is in response to novel local aspects of the tumor microenvironment.

Legumain is found on the CT26 tumor cell surfaces where it is effectively removed by 30 min collagenase digest at 57°C (FIG. 13B). These data indicate that legumain is found on the cell surface. This is surprising not only because legumain was previously identified as being an intracellular protein but also because legumain has no transmembrane domain and no prior evidence exists that points to secretion or plasmalemma localization of this lysosomal protease.

Flow cytometry was used to further analyze the extracelluar localization of legumain in tumors and normal organs. Flow cytometry permitted analysis of cell surface legumain in single cell suspensions prepared from tumors, bone marrow, spleen, and kidney, as well as cultured tumor cells. Despite demonstrable intracellular legumain by renal tubular epithelial cells, less than 2% of isolated viable cells were very weakly positive for cell surface legumain. Spleen cells have considerably less legumain than renal cells; however, approximately 5% of spleen cells are weakly positive for cell surface legumain. Furthermore, cell surface legumain is not detectable on cells derived from bone marrow nor is it found on cultured CT26 cells. In contrast, 40% of intact viable cells derived from *in vivo* CT26 tumors were strongly positive for cell surface legumain (FIG. 13C). A similar pattern was observed for all tumors examined (data not shown) indicating that cell surface and extracelluar legumain is uniquely abundant only in tumors.

Legumain is also expressed by tumor vascular endothelial cells (FIG. 14A). However, legumain is also expressed by on tumor-associated macrophages (TAMs) as observed by dual staining of tissue sections from CT26 tumors with anti-legumain and anti-CD68 antibodies (FIG. 14B). Secreted legumain is present in the tumor stroma associating with extracellular matrix proteins, such as collagen I (FIG. 14C). Legumain expression was not detected in normal peripheral blood monocytes. Using flow cytometry legumain was also found on the surface of viable endothelial cells and tumor-associated macrophages using both anti-legumain antibody and anti-CD31 antibody or anti-CD14 antibody respectively (FIG. 14D). Interestingly, legumain on endothelial cell and tumor associated macrophage surfaces is resistant to removal by

collagenase (FIG. 14E), suggesting a mode of cell surface association that is distinct from that of tumor cells where legumain is removed by collagen.

LEG-3 is activated by tumoral legumain. The ability of the cell impermeable targeting tumor microenvironment prodrug LEG-3 (FIG. 15A) to kill tumor cells was evaluated first in cell culture (FIG. 15B). The median effective concentration, which is the amount of LEG-3 or Dox required for 50% cell death (EC_{50}), was determined for both legumain-transfected and control non-producing cells (Table 4).

Table 4: EC50 (μM) of Dox and LEG-3.

	293 cells	**Legumain+293 cells**	**HTP cells**
Dox	1.2	1.4	1.5
LEG-3	>100	3.2	9.6
LEG-3 + cystatin	>100	64.4	59.2
LEG-4	>100	>100	>100

LEG-3 was virtually non-cytotoxic to cells not expressing legumain. However, for cells that were transfected with legumain cDNA and express cell surface legumain, cytotoxicity was significant and EC_{50} levels were close to those observed for Dox, indicating an efficient conversion of the LEG-3 prodrug.

The requirements for activation were demonstrated using an alternate peptide sequence not hydrolyzed by legumain (FIG. 15A-B) and also by inhibition of LEG-3 activation following inhibition of legumain function (FIG. 15C).

LEG-3 is activated extracellularly. Tumor cell uptake of LEG-3 was compared to Dox. When added to cell cultures, Dox rapidly entered cells. In contrast, the LEG-3 remained extracellular, consistent with its observed lack of cytotoxicity. In contrast, when LEG-3 was added to legumain expressing cells in culture, the end product Dox was found in cells (FIG. 15D-E).

Legumain is selectively functional in the tumor microenvironment. The maximum tolerable dose (MTD) of Dox and a molar equivalent amount of

LEG-3 were given intravenously as a single intravenous bolus into mice bearing tumors after receiving CT26 tumor cells.

When Dox was administered, the plasma concentration very rapidly declined and then a low Dox concentration was detected that slowly cleared (FIG. 16A). There was significant Dox content in many tissues including tumor, heart, kidney, liver, and brain. These observations are consistent with observations by other investigators (Mosure et al., Cancer Chemother. Pharmacol. 40: 251-58 (1997); Dubois et al., Cancer Res. 62: 2327-31 (2002)).

In contrast, the initial decline of infused LEG-3 was much slower (FIG. 16A) than observed for Dox. Given the hydrophilic nature of LEG-3, such slow decline in LEG-3 plasma concentration may be attributed to reduced tissue uptake. LEG-3 concentrations in cardiac, liver, kidney, spleen and lung tissues were reduced when compared to Dox levels in those tissues (FIG. 16B). However, twelve hours post-infection, the content of LEG-3 was more than 10-fold greater in tumor tissues than that observed for Dox (FIG. 16B). Because of its reduced normal tissue uptake and its reduced toxicity, larger quantities of LEG-3 could be administered, which resulted in higher drug content in cells within tumors compared to that achieved for Dox administration.

To confirm the tissue distribution, Drug accumulation in tissues and tumor was visualized by Dox autofluorescence (FIG. 16C). These data further indicate that legumain is selectively found in the tumor microenvironment and that LEG-3 is processed to its cell permeable Leu-Dox product because Dox is detected within the cytoplasm of tumor cells after injection of LEG-3. Moreover, Dox appears to be further processed and translocated to the nucleus because intranuclear Dox fluorescence was detected in tumor cells of LEG-3 injected mice.

***In vivo* toxicity of LEG-3 and Dox.** LEG-3 is significantly less toxic than Dox when evaluated *in vivo*. When given in five repeated intravenous administrations, LEG-3 had a much higher cumulative MTD and a reduced LD_{50} compared to Dox (Tables 5-6). Thus, significantly more LEG-3 can be administered without toxic effects.

Table 5: *In vivo* toxicity of LEG-3 compared to DOX.
Estimated MTD and LD_{50} of Dox and LEG-3 (μmol/kg) in Balb/c male mice.

	Dox		LEG-3	
	MTD	**LD_{50}**	**MTD**	**LD_{50}**
i.p.	9.8	17.2	>197.4	>197.4
i.p. 5×	2.8	3.4	98.7	>197.4
i.v.	18.2	25.8	>197.4	>197.4
i.v. 5×	3.2	5.1	74.0	197.4

Table 6: *In vivo* toxicity of LEG-3 compared to DOX.
Comparison of gross toxicity of mice treated with Dox and LEG-3.

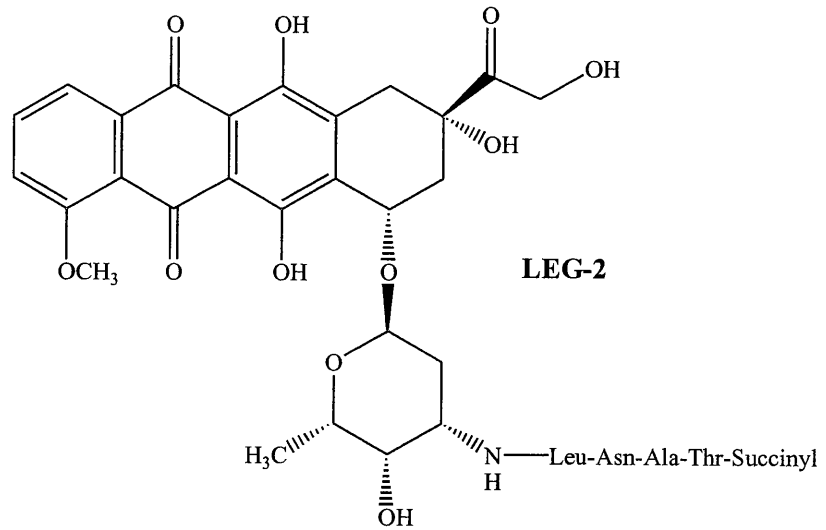
Controls						
	Average weight loss (%)	4				
Dox						
	Dose (μmol/kg)	1.72	5.4			
	Average weight loss (%)	24	28			
	Death (%)	35	75			
LEG-3	Dose (μmol/kg)	1.97	4.9	49.4	98.7	197.4
	Average weight loss (%)	3	5	7	8	16
	Death (%)	0	0	0	10	50

The cytotoxic effect of LEG-3 on peripheral blood leukocyte counts (WBC) was assessed and compared to the cytotoxic effects of Dox. There were no reductions of total WBC counts in mice treated six times in twelve days with 4.94 μmol/kg LEG-3 and only a slight reduction observed in mice treated with a 10-fold higher dose. In contrast, there was greater than 50% reduction in WBC counts in the group of mice receiving as little as 3.4 μmol/kg Dox on the same injection schedule (FIG. 17A). LEG-3 produced little evidence of myelosuppression compared to its parent compound Dox.

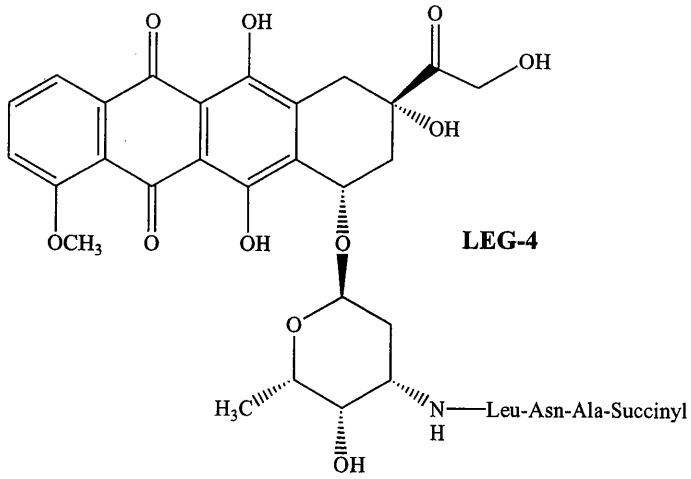
Bone marrow and cardiac toxicity of LEG-3 was also examined and compared to Dox. In cardiac tissue, mice treated with 49.4 μmol/kg of LEG-3 exhibited no histological evidence of cardiac toxicity. However, profound

cardiac myocyte vacuolization and cell death was observed in mice treated with Dox at concentrations of 3.4 μmol/kg (FIG. 17B). Moreover, cardiac myocytes from mice treated with Dox demonstrated marked apoptosis, whereas this was not observed for LEG-3 treated mice (FIG. 17B).

***In vivo* efficacy is dependent on legumain specific activity.** To further characterize the requirements for legumain activation of LEG-3 *in vivo*, two LEG-type compounds differing in the composition of the peptidyl element of the compound were evaluated. These LEG-type compounds were LEG-2 and LEG-4, shown below.



LEG-2



LEG-4

LEG-2 and LEG-4 were analyzed for *in vivo* efficacy in tumor bearing mice. The LEG-4 compound is cleaved by legumain (data not shown) and was

devoid of tumoricidal activity (FIG. 18A). However, LEG-2 and LEG-3 are cleaved by legumain and possessed demonstrable *in vivo* tumoricidal efficacy (FIG. 18B-D).

LEG-3 has effective tumoricidal activity against diverse tumors *in vivo*.

The therapeutic efficacy of LEG-3 was evaluated in both syngeneic rodent tumor models and human tumor xenograft models. When administered six times in 12 days at a well tolerated dose (49.4 μM/kg), LEG-3 effectively arrested growth of murine CT26 colon carcinoma in Balb/c mice and produced complete tumor eradication in some mice (FIG. 18C). A similar level of efficacy was also observed for the murine C1300 neuroblastoma in A/J mice (FIG. 18D), where LEG-3 treatment induced massive tumor cell death (FIG. 18C-D), tumor eradication and marked enhancement of survival (FIG. 18E-F).

The *in vivo* efficacy of LEG-3 on human tumor xenografts in athymic nu/nu mice was assessed and compared to Dox. As described above, legumain protein was not detectable in either HT1080 or MDA-PCa-2b cells when those cells were maintained in culture. However, robust legumain expression was observed by immunohistochemistry of *in vivo* tumors propagated from these cells. Indeed, LEG-3 produced potent tumoricidal activity against the HT1080 fibrosarcoma, a fast-growing tumor and a model tumor that is known to be sensitive to Dox therapy (FIG. 19A).

On the other hand, human prostate carcinomas are frequently resistant to Dox therapy. For example, an MDA-PCa-2b prostate carcinoma that is a known Dox resistant tumor (Letsch et al., Clin. Cancer Res. 9: 4505-13 (2003)), failed to respond to Dox *in vivo*. However, administration of LEG-3 led to complete growth arrest (FIG. 19B) of these Dox-insensitive prostrate tumor cells. LEG-3 frequently resulted in complete tumor eradication with marked enhancement of survival of the HT1080- as well as the MDA-PCa-2b-tumor bearing mice (FIG. 19C-D). Toxicity of LEG-3 based on weight loss and mortality was negligible (FIG. 19E).

Therefore, the cell impermeable targeting tumor microenvironment activated prodrug (TMEAP) strategy described herein was highly effective against a variety of tumor types *in vivo*.

The present prodrugs include a peptidyl targeting agent for legumain which exhibits specificity in its catalytic function at a pH <6.8 and is selectively expressed extracellularly in the tumor microenvironment. Due to its extracellular activity, legumain can convert an effectively designed cell impermeable prodrug (e.g. LEG-3) to a cell permeable prodrug (Leu-Dox) which in turn is converted to the active tumoricidal end product Dox and translocated to cell nuclei for induction of cell death.

There are distinct advantages to this strategy in that tissue uptake can occur only in the tumor or comparable pathologic microenvironment. To achieve continuous growth and remodeling, the tumor microenvironment is enriched with a variety of proteases. There are nearly five hundred proteases identified so far in the human genome, and many have been associated with the local tumor microenvironment and appear important for tumor invasion and metastasis. Drug access to solid tumors is relatively efficient, limited only by diffusion barriers. Compounds like LEG-3 can therefore be converted and activated in the tumor microenvironment so that they produce substantial bystander effects not only on the tumor cells but also the associated endothelial and stromal cells. Whereas, most cytotoxic drugs are designed to be cell permeable, the present prodrugs have diminished cell permeability, increased hydrophilic properties, and increased drug solubility to minimize tissue uptake of LEG-3. This correlates with the slower clearance from the blood as well as greatly diminished tissue accumulation. In contrast, tumor cells exhibit increased uptake of the present prodrugs.

Thus, the invention provides anti-tumor prodrugs with improved properties.

Example 7: Selective depletion of macrophages delays tumor growth

An established procedure was tested to observe the effects of selectively killing macrophages upon macrophage on tumor development in 4T1 breast cancer models. Cl_2MDP liposomes were used as agents that specifically target and kill macrophages.

Mice (Balb c, n=4) were treated with Cl_2MDP liposomes while control mice (n=4) were untreated. All mice were then challenged with syngeneic 4T1 breast cancer cells.

The Cl_2MDP liposome treatment resulted macrophage depletion and a significant delay of tumor growth. In particular, untreated cancer cells formed 4T1 breast cell tumors in about 7 days. In contrast, Cl_2MDP liposome treatment delayed tumor development substantially to about 45 days after tumor cell injection.

Therefore, macrophages have an important role of in the development of tumors.

Example 8: Legumain activated prodrug selectively target tumor-associated macrophages

This Example illustrates that liposome formulations of the inventive prodrugs can selectively deplete tumor associated macrophages (TAM) from tumors.

Methods

LEG-3 was synthesized as described in previous Examples.

To prepare liposome encapsulated LEG-3, stock solutions of phosphatidylcholine and cholesterol were prepared by dissolving phosphatidylcholine (PC) and cholesterol (0.8 mg) in 1 mL of chloroform each. These stock solutions can be stored in -20°C under N_2 with the cap tightened and sealed with parafilm. The phospholipid layer was prepared in a molar ratio of 98 phosphatidylcholine: 2 cholesterol by adding 155 µL of phosphatidylcholoine to 193 µL of cholesterol in a 15 x 85 mm glass tube. The lipid mixture was placed under a gentle stream of N_2 gas until all chloroform was evaporated. To this mixture was added between 0.5 - 1 ml of Leg-3 compound (1 mg/100 µl, 0.9% saline). The mixture was incubated at room temperature for at least 30 minutes, then vortexed gently and intermittently until the phospholipid layer went into solution and was no longer seen on the bottom of tube. The mixture was then sonicated for 10-20 minutes, checking periodically to see if the solution was less opaque. The preparation was then transferred to a 1.5 mL tube and centrifuged at

10,000 g for 10 minutes. A pink lipid layer formed at the top of the tube. This layer contained the liposomes. This liposome-containing layer was transferred to another 1.5 mL tube and washed 2-3 times with sterile 0.9% saline using centrifugation at 25,000 g for 10 minutes at 4°C.

The liposomes were resuspended in sterile 0.9% saline prior to injection into mice.

Results

Unlike the Cl_2MDP liposome, administration of LEG-3 liposomes selectively killed tumor-associated macrophages (TAMs) without harm M1 type of macrophages. In particular, CD14 positive macrophages were not depleted from the spleen in mice that were treated with LEG-3 liposomes. Therefore, the normal innate immune functions of macrophages were unaffected.

In contrast, the LEG-3 liposome preparation selectively depleted TAMs in tumors (FIG. 20A). This TAM depletion resulted in significant reduction of angiogenic growth factors (FIG. 20B and C). Treatment with liposome encapsulated LEG-3 at low dosage (5umol/kg) also resulted in significant growth suppression of 4T1 tumor in mice (FIG. 21). This effect was substantially due to the depletion of macrophages and the resulting anti-angiogenic effect. Therefore, the LEG-3 prodrug kills TAM effectively and reduces the levels of angiogenic factors in tumors.

However, some evidence suggested that TAM infiltrates recover quickly and so does the production of angiogenic factors. These data suggest that to effectively attack tumors targeting TAM, a metronomic dosing of the TME activated prodrug may be necessary.

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All patents and publications referenced or mentioned herein are indicative of the levels of skill of those skilled in the art to which the invention pertains, and each such referenced patent or publication is hereby incorporated by reference to the same extent as if it had been incorporated by reference in its entirety individually or set forth herein in its entirety. Applicants reserve the right to physically incorporate into this specification any and all materials and information from any such cited patents or publications.

The specific methods and compositions described herein are representative of preferred embodiments and are exemplary and not intended as limitations on the scope of the invention. Other objects, aspects, and embodiments will occur to those skilled in the art upon consideration of this specification, and are encompassed within the spirit of the invention as defined by the scope of the claims. It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention. The invention illustratively described herein suitably may be practiced in the absence of any element or elements, or limitation or limitations, which is not specifically disclosed herein as essential. The methods and processes illustratively described herein suitably may be practiced in differing orders of steps, and that they are not necessarily restricted to the orders of steps indicated herein or in the claims. As used herein and in the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, a reference to "a host cell" includes a plurality (for example, a culture or population) of such host cells, and so forth.

Under no circumstances may the patent be interpreted to be limited to the specific examples or embodiments or methods specifically disclosed herein. Under no circumstances may the patent be interpreted to be limited by any statement made by any Examiner or any other official or employee of the Patent and Trademark Office unless such statement is specifically and without qualification or reservation expressly adopted in a responsive writing by Applicants.

The terms and expressions that have been employed are used as terms of description and not of limitation, and there is no intent in the use of such terms and expressions to exclude any equivalent of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention as claimed. Thus, it will be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

The following statements of the invention are intended to characterize possible elements of the invention according to the foregoing description given in the specification. Because this application is a provisional application, these statements may become changed upon preparation and filing of a nonprovisional application. Such changes are not intended to affect the scope of equivalents according to the claims issuing from the nonprovisional application, if such changes occur. According to 35 U.S.C. § 111(b), claims are not required for a provisional application. Consequently, the statements of the invention cannot be interpreted to be claims pursuant to 35 U.S.C. § 112.

FORMAL CLAIM:

1. An anti-legumain antibody that binds to an epitope consisting essentially of CGMKRASSPVPLPP (SEQ ID NO:16).

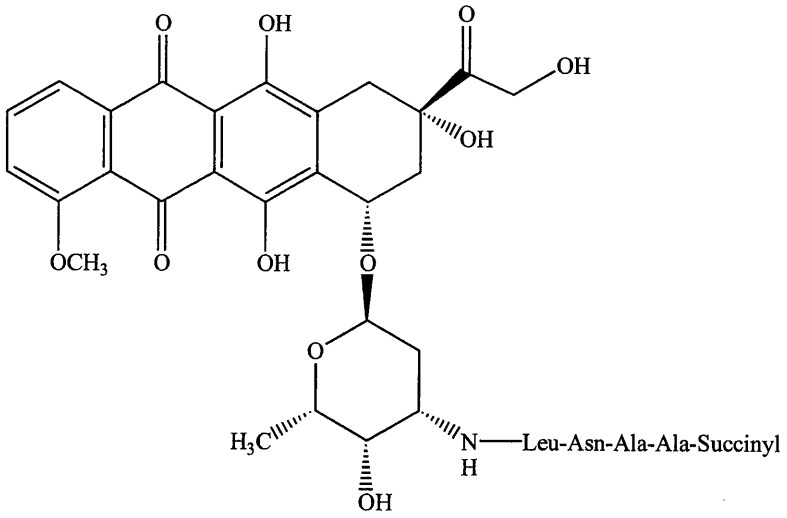
2. A method of inhibiting angiogenesis from mammalian cells comprising contacting the cells with an effective amount of an asparaginyl endopeptidase inhibitor.

3. The method of claim 2, wherein the asparaginyl endopeptidase inhibitor is Cbz-Ala-Ala-AzaAsn-(S,S)-EPCOOEt, Cbz-Ala-Ala-AzaAsn-CH=CH-COOEt, Cbz-Ala-Ala-AzaAsn-CH=CH-COOBzl or Cbz-Ala-Ala-AzaAsn-CH=CH-CON(CH3)Bzl, where Cbz is benzyloxycarbonyl, Et is ethyl and Bzl is benzyl.

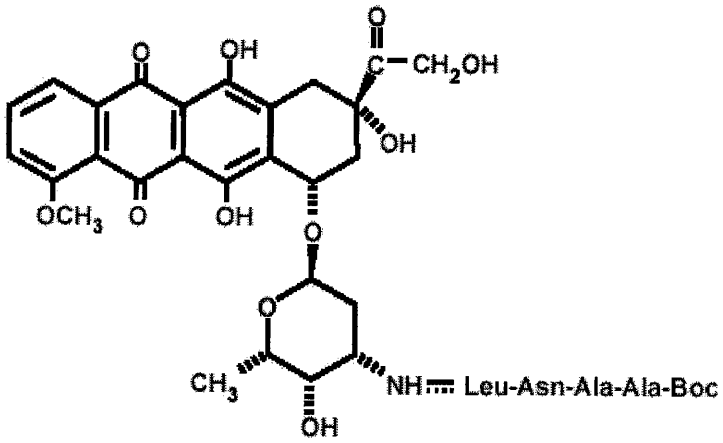
4. The method of claim 2, wherein asparaginyl endopeptidase inhibitor is and anti-legumain antibody.

5. The method of claim 4, wherein the anti-legumain antibody binds to an epitope consisting essentially of CGMKRASSPVPLPP (SEQ ID NO:16).

6. A composition comprising a liposomal carrier and a therapeutically effective amount of a water-soluble prodrug, wherein the water-soluble prodrug is a compound of the following structure:



or



or a combination thereof.

STATEMENTS OF THE INVENTION:

1. A method of inhibiting angiogenesis in a mammal comprising administering to the mammal a therapeutically effective amount of an asparaginyl endopeptidase inhibitor.

2. The method of statement 1, wherein the angiogenesis is associated with a tumor.

3. The method of statement 1, wherein the asparaginyl endopeptidase inhibitor is Cbz-Ala-Ala-AzaAsn-(S,S)-EPCOOEt, Cbz-Ala-Ala-AzaAsn-CH=CH-COOEt, Cbz-Ala-Ala-AzaAsn-CH=CH-COOBzl or Cbz-Ala-Ala-AzaAsn-CH=CH-CON(CH3)Bzl, where Cbz is benzyloxycarbonyl, Et is ethyl and Bzl is benzyl.

4. The method of statement 1, wherein the asparaginyl endopeptidase inhibitor is a peptide comprising Ala-Leu-β-Asn-Ala-Ala (SEQ ID NO:15).

5. The method of statement 1, wherein the asparaginyl endopeptidase inhibitor is cystatin C or stefin B.

6. The method of statement 1, wherein the cystatin C is a mutated cystatin C.

7. The method of statement 1, wherein the asparaginyl endopeptidase inhibitor is a compound of formula formula **III**, **IV** or **V**:

$$\text{Hyd-(Xaa4)}_n\text{-Asn-CHO} \qquad \textbf{III}$$

Xaa4-Asn-Y **IV**

Xaa4-azaAsn-Y **V**

wherein:

Hyd is a hydrophilic group;

n is an integer of about 2 to about 5;

Xaa4 is an amino acid or an amino acid mimetic;

Y is alkyl, alkenyl, an epoxide or a Michael acceptor optionally substituted with 1-3 halo or hydroxy, alkylamino, dialkylamino, alkyldialkylamino, or cycloalkyl, alkylcycloalkyl, alkenylcycloalkyl, aryl; (C_5 -C_{12})arylalkyl or (C_5 -C_{12})arylalkenyl,

wherein the aryl groups of the arylalky or arylalkenyl can be 0-4 heteroatoms selected from N, O and S, and are optionally substituted with halo, cyano, nitro, haloalkyl, amino, aminoalkyl, dialkylamino, alkyl, alkenyl, alkynyl, alkoxy, haloalkoxy, carboxyl, carboalkoxy, alkylcarboxamide, (C_5 -C_6)aryl, --O-(C_5 -C_6)aryl, arylcarboxamide, alkylthio or haloalkylthio; and

wherein the inhibitor is capable of binding to legumain.

8. The method of statement 1, wherein the asparaginyl endopeptidase inhibitor is and anti-legumain antibody.

9. The method of statement 8, wherein the anti-legumain antibody binds to an epitope consisting essentially of CGMKRASSPVPLPP (SEQ ID NO:16).

10. A method of inhibiting angiogenesis in a mammal comprising administering a therapeutically effective amount of a water-soluble prodrug compound, comprising a drug molecule linked to a legumain peptide substrate, wherein the peptide has an amino acid sequence comprising at least two linked amino acids, wherein at least one of the two linked amino acids is Asn, and wherein legumain cleaves the peptide at the link between the Asn and another amino acid to generate an active drug from the prodrug.

11. The method of statement 10, wherein the prodrug is substantially non-toxic to non-legumain expressing animal cells.

12. The method of statement 10, wherein the drug is a cytotoxin.

13. The method of statement 12, wherein the cytotoxin comprises aldesleukin, asparaginase, bleomycin sulfate, camptothecin, carboplatin, carmustine, cisplatin, cladribine, lyophilized cyclophosphamide, non-lyophilized cyclophosphamide, cytarabine, dacarbazine, dactinomycin, daunorubicin, diethyistilbestrol, epoetin alfa, esperamycin, etidronate, etoposide, filgrastim, floxuridine, fludarabine phosphate, fluorouracil, goserelin, granisetron hydrochloride, idarubicin, ifosfamide, immune globulin, interferon alpha-2a, interferon alpha-2b, leucovorin calcium, leuprolide, levamisole, mechiorethamine, medroxyprogesterone, melphalan, methotrexate, mitomycin, mitoxantrone, octreotide, ondansetron hydrochloride, paclitaxel, pamidronate, disodium, pegaspargase, plicamycin, sargramostim, streptozocin, taxol, thiotepa, teniposide, vinblastine, or vincristine.

14. The method of statement 10, wherein the drug is doxorubicin or paclitaxel.

15. The method of statement 10, wherein the prodrug compound comprises SEQ ID NO:3:

$$\text{Hyd-(Xaa1)}_n\text{-Xaa2-Asn-(Xaa3)-drug}$$

wherein:

Hyd is a hydrophilic protecting group;

n is an integer of about 0 to about 50;

Xaa1 and Xaa2 are separately any amino acid;

Xaa3 is either nothing or an amino acid that has no substantial effect on the activity of the drug; and

the drug is a drug whose action is diminished or blocked by attachment of a peptide to the drug.

16. The method of statement 15, wherein the Hyd group is succinyl.

17. The method of statement 10, wherein the amino acid sequence comprises Asn-Leu, Ala-Asn-Leu, or Thr-Asn-Leu.

18. The method of statement 10, wherein the amino acid sequence comprises Boc-Ala-Ala-Asn-Leu (SEQ ID NO:4) or Ala-Thr-Asn-Leu (SEQ ID NO:6).

19. The method of statement 10, wherein the amino acid sequence comprises Ala-Ala-Asn-Leu (SEQ ID NO:5).

20. The method of statement 10, wherein the amino acid sequence comprises succinyl-Ala-Ala-Asn-Leu-doxorubicin (SEQ ID NO:8).

21. The method of statement 10, wherein the peptide further comprises a hydrophilic protecting group.

22. The method of statement 21, wherein the protecting group is an amino protecting group.

23. The method of statement 21, wherein the protecting group is succinyl.

24. The method of statement 21, wherein the protecting group is *t*-butoxycarbonyl.

25. The method of statement 10, wherein the peptide further comprises an *N*-*β*-alanyl terminus.

26. The method of statement 10, wherein the compound comprises N-(-*t*-Butoxycarbonyl-Ala-Thr-Asn-Leu)doxorubicin (SEQ ID NO:9), N-(-*t*-Butoxycarbonyl-Ala-Asn-Leu)doxorubicin (SEQ ID NO:11), N-(-*t*-Butoxycarbonyl-Thr-Leu)doxorubicin (SEQ ID NO:13), or N-(-*t*-Butoxycarbonyl-L-Ala-L-Ala-L-Asn)taxel.

27. The method of statement 10, wherein the compound comprises N-(Succinyl-Ala-Thr-Asn-Leu)doxorubicin (SEQ ID NO:10), N-(Succinyl-Ala-Asn-Leu)doxorubicin (SEQ ID NO:12), N-(Succinyl-Thr-Leu)doxorubicin (SEQ ID NO:14) or N-(succinyl-L-Ala-L-Ala-L-Asn)taxel.

28. The method of statement 10, wherein the angiogenesis is associated with tumorigenesis.

29. The method of statement 28, wherein the tumorigenesis further involves metastasis of cancer cells.

30. The method of statement 28, wherein the tumorigenesis involves one or more tumors of breast, colon, lung, prostate, ovarian, central nervous system, or lymph tissues.

31. The method of statement 10, wherein the angiogenesis is associated with autoimmune deficiency syndrome-associated Kaposi's sarcoma, cancer of the adrenal cortex, cancer of the cervix, cancer of the endometrium, cancer of the esophagus, cancer of the head and neck, cancer of the liver, cancer of the pancreas, cancer of the prostate, cancer of the thymus, carcinoid tumors, chronic lymphocytic leukemia, Ewing's sarcoma, gestational trophoblastic tumors, hepatoblastoma, multiple myeloma, non-small cell lung cancer, retinoblastoma, or tumors in the ovaries.

32. The method of statement 10, wherein the angiogenesis is associated with colon carcinoma, breast carcinoma, neuroblastoma, fibrosarcoma or prostate carcinoma.

33. The method of statement 10, wherein the prodrug is formulated as a liposome:prodrug formulation.

34. A method for treating a mammal having, or suspected of having, cancer, comprising administering to the mammal a therapeutically effective amount of a agent that binds to a cell surface legumain:integrin complex and inhibits legumain activity.

35. A method for treating a mammal having, or suspected of having, cancer, comprising administering to the mammal a therapeutically effective amount of a water-soluble prodrug that binds to a cell surface legumain:integrin complex, wherein the prodrug includes a drug that is activated by legumain cleavage.

36. The method of statement 34 or 35, wherein the integrin substantially increases legumain activity.

37. The method of statement 34 or 35, wherein the integrin increases legumain activity by about 10-fold to about 200-fold.

38. The method of statement 34 or 35, wherein the integrin substantially increases legumain activity at about pH 6.0 to about pH 6.5.

39. The method of statement 34 or 35, wherein the cancer is a solid cancer.

40. The method of statement 34 or 35, wherein the cancer is metastatic or invasive cancer.

41. The method of statement 34 or 35, wherein the cancer is breast cancer, colon cancer, lung cancer, prostate cancer, ovarian cancer, cancer of the central nervous system, lymphoma, or melanoma.

42. The method of statement 34 or 35, wherein the cancer is autoimmune deficiency syndrome-associated Kaposi's sarcoma, cancer of the adrenal cortex, cancer of the cervix, cancer of the endometrium, cancer of the esophagus, cancer of the head and neck, cancer of the liver, cancer of the pancreas, cancer of the prostate, cancer of the thymus, carcinoid tumors, chronic lymphocytic leukemia, Ewing's sarcoma, gestational trophoblastic tumors, hepatoblastoma, multiple myeloma, non-small cell lung cancer, retinoblastoma, or tumors in the ovaries.

43. The method of statement 34 or 35, wherein the cancer is colon carcinoma, breast carcinoma, neuroblastoma, fibrosarcoma or prostate carcinoma.

44. The method of statement 34, wherein the agent is Cbz-Ala-Ala-AzaAsn-(S,S)-EPCOOEt, Cbz-Ala-Ala-AzaAsn-CH=CH-COOEt, Cbz-Ala-Ala-AzaAsn-CH=CH-COOBzl or Cbz-Ala-Ala-AzaAsn-CH=CH-CON(CH3)Bzl, where Cbz is benzyloxycarbonyl, Et is ethyl and Bzl is benzyl.

45. The method of statement 34, wherein the agent is an anti-legumain antibody that binds to an epitope consisting essentially of CGMKRASSPVPLPP (SEQ ID NO:16).

46. The method of statement 34, wherein the agent is a mutated cystatin.

47. The method of statement 35, wherein the water-soluble prodrug comprises a drug molecule linked to a legumain peptide substrate, wherein the peptide has an amino acid sequence comprising at least two linked amino acids, wherein at least one of the two linked amino acids is Asn, and wherein legumain cleaves the peptide at the link between the Asn and another amino acid to generate an active drug from the prodrug.

48. The method of statement 47, wherein the prodrug is formulated is a liposome:prodrug formulation.

49. A method for inhibiting activation of metalloprotease-2 and cathepsin comprising contacting the metalloprotease-2 or cathepsin with an agent that inhibits legumain.

50. The method of statement 49, wherein the metalloprotease-2 or cathepsin are present in a mammal.

51. A method for inhibiting angiogenesis, tumor invasion or metastasis in a mammal comprising inhibiting activation of metalloprotease-2 or cathepsin by administering to the mammal an agent that inhibits legumain.

52. A method for inhibiting tumor-associated macrophages in a mammal comprising administering to the mammal a water-soluble prodrug, wherein the water-soluble prodrug comprises a drug molecule linked to a legumain peptide substrate, wherein the peptide has an amino acid sequence comprising at least two linked amino acids, wherein at least one of the two linked amino acids is Asn, and wherein legumain cleaves the peptide at the link between the Asn and another amino acid to generate an active drug from the prodrug.

53. The method of claim 52, wherin the prodrug is formulated as a liposome:prodrug formuation.

54. A method for detecting or monitoring metastatic cancer, comprising contacting a test tissue suspected of comprising cancer cells with of an anti-legumain antibody that specifically binds to a legumain epitope consisting essentially of CGMKRASSPVPLPP (SEQ ID NO:16), and detecting whether the antibody binds to the test tissue.

55. The method of statement 54, which further comprises quantifying and comparing amounts of the antibody bound to the test tissue with amounts of the antibody bound to a control tissue that is not cancerous.

56. The method of statement 54, wherein the antibody is linked to a detectable label.

57. A method for diagnosing cancer in an animal, comprising administering to the animal an anti-legumain antibody that specifically binds to a legumain epitope consisting essentially of CGMKRASSPVPLPP (SEQ ID NO:16), and detecting whether the antibody accumulates in a tissue.

58. The method of statement 57, wherein the antibody is linked to a detectable label.

59. A method for inhibiting tumor cell invasion, tumor cell metastasis or tumor angiogenesis in a mammal comprising administering to the mammal an amount of a prodrug that is sufficient to inhibit tumor stromal cells from producing angiogenic factors and growth factors.

60. The method of statement 59, wherein the prodrug is activated by a legumain:integrin complex.

61. The method of statement 59, wherein the stromal cells are tumor associsted macrophages.

62. The method of statement 59, wherein the stromal cells are angiogenic endothelial cells.

63. A composition comprising a liposomal carrier and a therapeutically effective amount of a water-soluble prodrug, wherein the water-soluble prodrug comprises a drug molecule linked to a legumain peptide substrate, wherein the peptide has an amino acid sequence comprising at least two linked amino acids, wherein at least one of the two linked amino acids is Asn, and wherein legumain cleaves the peptide at the bond between the Asn and another amino acid to generate an active drug from the prodrug.

Abstract of the Invention

The present invention relates to new compositions and methods useful for preventing, treating and diagnosing metastatic and/or invasive cancer and undesirable angiogenesis. For example, the invention relates to asparaginyl endopeptidase inhibitors, prodrugs activated in the tumor microenvironment and methods for using those inhibitors and prodrugs to inhibit angiogenesis and tumor cell invasion.